Universidade de Aveiro
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Departamento de Biologia

FILIPA ISABEL PEREIRA DIAS

CARACTERIZAÇÃO DA EXPRESSÃO GENÉTICA DA VIA DO FOLATO EM CARRAÇAS

GENE EXPRESSION CHARACTERIZATION OF TICKS' FOLATE PATHWAY

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Ana Domingos, Investigadora do Instituto de Higiene e Medicina Tropical da Universidade Nova de Lisboa (IHMT-UNL) e co-orientação da Doutora Sónia Mendo, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho aos meus pais, pelo seu amor incondicional e por acreditarem em mim quando eu própria não acreditei.

o júri

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palavras-chave

Doenças transmitidas por carraças, via do folato, qPCR, expressão diferencial, RNAi

resumo

As doenças transmitidas por carraças têm um grande impacto mundial na saúde humana e animal, sendo também responsáveis por um grande fardo económico nas indústrias pecuárias. Desta forma, há uma necessidade de aumentar e melhorar a nossa compressão das carraças como vetores e dos agentes patogénicos que estas transmitem para o desenvolvimento de medidas economicamente viáveis para o seu controlo e erradicação. Vacinas com a capacidade de afetar várias espécies de carraças ou capazes de bloquear a transmissão dos agentes patogénicos são uma abordagem promissora para este problema. No entanto, o desenvolvimento de vacinas é altamente dependente da seleção de antigénios apropriados. A via do folato é um dos alvos para o controlo e tratamento da malária, sendo interessante pelos seus amplos, mas essenciais papeis na sobrevivência dos organismos, incluindo a biossíntese de purinas, pirimidinas, tetrahidrobiopterina, entre muitos outros. Aqui, é estudado como as interações com os parasitas/bactérias modelam a expressão da via do folato nas carraças e o potencial destes alvos como antigénios candidatos para o desenvolvimento de vacinas. A identificação de genes da via do folato foi realizada através de PCR e qPCR em três espécies de carraça do género *Rhipicephalus* (*Rhipicephalus annulatus, Rhipicephalus bursa* e *Rhipicephalus sanguineus*) e também na linha celular de *Ixodes scapularis* (IDE8). A expressão diferencial destes genes foi analisada entre amostras não infetadas e infetadas em quatro sistemas biológicos (*R. annulatus – Babesia bigemina, R. bursa – Babesia ovis, R. sanguineus – Ehrlichia canis*, IDE8 – *E. canis*) seguida da seleção de alvos para análise funcional *in vitro* através de RNA de interferência. Para o ensaio de silenciamento, RNA em cadeia dupla foi inoculado em células IDE8 não infectas e infetadas com *E. canis*. Amostras foram coletadas em três tempos para avaliar o efeito do silenciamento do gene na morfologia das células e na invasão e replicação das bactérias nas células de carraça. Foi possível identificar cinco genes em *R. annulatus* e apenas três nos restantes sistemas biológicos. No geral, foi observado um aumento da expressão dos genes em resposta a infeção, apesar de nem sempre ser estatisticamente significativo. O gene que codifica para a enzima GTP cyclohydrolase I (GCH-I) foi selecionado para o ensaio de silenciamento por apresentar a maior diferença de expressão ($p < 0.01$) na maioria dos sistemas biológicos testados. Silenciamento deste gene na linha celular IDE8 não mostrou nenhuma alteração na morfologia das células e nenhum efeito na invasão e multiplicação da bactéria nas células. Estes resultados sugerem uma modulação da expressão génica da via do folato seja como resposta da carraça ao organismo invasor ou como manipulação da maquinaria celular da carraça pelo patogénio para sua vantagem, sendo alvos interessantes para mais estudos.

keywords Tick-borne diseases, folate pathways, qPCR, differential expression, RNAi

abstract

Ticks and tick-borne diseases have a high impact in human and animal health worldwide, being also responsible for a great economic burden in the livestock industry. As such, there is need to increase and improve our understanding of the tick vectors and the pathogens they transmit for the development of cost-effective measures of control and eradication. Vaccines with the capacity to target several tick species and/or capable to block pathogen transmission are a promising approach for this problem. However, vaccine development is highly dependent on the selection of appropriate antigens. The folate pathway is one of the targets in the control and treatment of malaria being interesting for its broad but essential roles in organism survival, including biosynthesis of purines, pyrimidines, tetrahydrobiopterin, between many others. Here, we study how parasites/bacteria interactions modulate the expression of the folate pathway in the tick vector and the potential of these targets as candidate antigens for vaccine development. Folate pathway gene identification was performed by PCR and qPCR in three Rhipicephalus tick species (*Rhipicephalus annulatus*, *Rhipicephalus bursa* and *Rhipicephalus sanguineus*) and also in the *Ixodes scapularis* tick cell line (IDE8). Differential expression of these genes was analysed between uninfected and pathogen infected samples in four biological systems (*R. annulatus* – *Babesia bigemina*, *R. bursa* – *Babesia ovis*, *R. sanguineus* – *Ehrlichia canis*, IDE8 – *E. canis*) followed by target selection for *in vitro* functional analysis by RNA interference. For the silencing assay, double stranded RNA was inoculated in uninfected and *E. canis* - infected IDE8 cells. Samples were collected in three time points to evaluate gene knockdown effect on cell morphology and bacterial invasion and replication in tick cells. It was possible to identity five genes in *R. annulatus* and only three in the other biological systems. Overall, an increase in gene expression was observed in response to infection, however not always statistically significative. The gene encoding for GTP cyclohydrolase I (GCH-I) was selected for the silencing assay for showing the largest fold-change ($p < 0.01$) in the majority of the tested biological systems. Silencing of this gene in the IDE8 cell line showed no alteration in tick cell morphology and no effect on the invasion and multiplication of the bacteria in the cells. These results suggest gene expression modulation of folate pathway either as a tick response to an invader or manipulation of the tick cell machinery by the pathogens to their advantage, being interesting targets for further studies.

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List of Abbreviations

AICARFT	Phosphoribosylaminoimidazolecarboxamide Formyltransferase
ALDH	Aldehyde Dehydrogenase
AMT	Aminomethyltransferase
AP	Alkaline Phosphatase
BH4	Tetrahydrobiopterin
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
ca.	Circa
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CO1	Cytochrome C Oxidase Subunit 1
DHF	Dihydrofolate
DHPR	Dihydropteridine Reductase
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dsb	Disulfide Bond Formation Protein Gene
dsRNA	Double Stranded RNA
dTMP	Deoxythymidine Monophosphate
dUMP	Deoxyuridine Monophosphate
E.C	Enzyme Commission
ELF	Elongation Factor
FPGS	Folylpolyglutamate Synthase
FTCD	Glutamate Formiminotransferase
GARFT	Phosphoribosylglycinamide Formyltransferase
GCH-I	GTP Cyclohydrolase I
GH	Gamma-Glutamyl Hydrolase
HME	Human Monocytic Ehrlichiosis
IFA	Indirect Florescence Antibody Assay
IHMT	Instituto de Higiene e Medicina Tropical
KEGG	Kyoto Encyclopedia of Genes and Genomes
L15B	Leibovitz's L15 Modified Medium
MCBPP1	Molybdenum Cofactor Biosynthesis Pathway Protein 1
MCBPP2	Molybdenum Cofactor Biosynthesis Pathway Protein 2
MCS	Molybdenum Cofactor Sulfurtransferase
MFT	Methionyl-tRNA Formyltransferase
MPTS	Molybdopterin Synthase
mRNA	Messenger RNA
MSLS	Molybdopterin Synthase Large Subunit
MTHFC	Methenyltetrahydrofolate Cyclohydrolase
MTHFS	5-Formyltetrahydrofolate Cyclo-Ligase
mya	Million Years Ago
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Center for Biotechnology Information
NO	Nitric Oxide
pABA	Para-aminobenzoic Acid

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pre-miRNA	Pre-microRNA
PTPS	6-Pyruvoyltetrahydropterin Synthase
qPCR	Quantitative Real-time Polymerase Chain Reaction
rDNA	Ribosomal DNA
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
RNA-seq	RNA Sequencing
SAM	S-Adenosylmethionine
SG	Salivary Gland
SHMT	Serine Hydroxymethyltransferase
shRNA	Small Hairpin RNA
siRNA	Short Interference RNA
sp.	Specie
spp.	Species (Plural)
SPR	Sepiapterin Reductase
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TBD	Tick Borne Diseases
TBE	Tris-Borate-EDTA Buffer
THF	Tetrahydrofolate
TS	Thymidylate Synthase
USA	United States of America
v/v	Volume per Volume
w/v	Weight per Volume
β2m	Beta-2 Microglobulin

1. Introduction

Ticks are the blood feeding ectoparasites that can transmit the greatest variety of pathogens from all arthropod groups (Sonenshine & Roe, 2014). Between the nearly 900 species reported worldwide they have been described to be able to infest not only mammals but also birds, reptiles and amphibians. This diversified selection of hosts and pathogens associated with the vast geographical distribution of ticks contributes to the high impact tick-borne diseases (TBDs) have on human and animal health (Jongejan & Uilenberg, 2004).

Lyme, Rocky Mountain spotted fever, Mediterranean spotted fever and human granulocytic anaplasmosis are some examples of the most important human TBDs, while babesiosis, theileriosis, anaplasmosis and ehrlichiosis severely affect livestock and companion animals (Sonenshine & Roe, 2014). However, ticks can be a severe threat on their own causing host paralysis and, when in large numbers, can reduce weight gain and milk production, damage hides or even lead to abortion in livestock (Sonenshine & Roe, 2014). As such, they heavily affect farming communities in Africa, Asia and Latin America, causing great economic losses with the application of unsuccessful vaccines and acaricide treatments, loss of animals and derived products (Jongejan & Uilenberg, 2004).

The increasing incidence of infectious TBDs can result from a vast number of factors such as: micro and macro climate changes, which affect the geographical distribution of the vector; alteration in human behaviour, like occupation of rural areas and increased contact with vegetation; introduction of domestic animals in new areas or changes in the wildlife populations (Heyman et al., 2010; Anderson & Magnarelli, 2008; Wikel, 2018). Therefore, efficient prevention and control of these diseases must be based on multidisciplinary approaches, as suggested by the "One Health" program, integrating professionals from human and animal health, environment scientists and experts from other sectors ("WHO | One Health," 2017).

With the increase of emerging and re-emerging TBDs in the last decades (Hook, Nelson & Mead, 2015; Mansfield, Jizhou, Phipps & Johnson, 2017; Socolovschi, Mediannikov, Raoult & Parola, 2009) there is a present need for the development of cost-effective and environmental friendly strategies to control, not only the vector but also the pathogens they transmit. To fill these requirements, there has been a rise into the design of

vaccines that target the host-tick-pathogen interface (de la Fuente & Contreras, 2015). The key factor for such approach relies on the selection of promising antigens with important biological roles (de la Fuente, Kopáček, Lew-Tabo & Maritz-Olivier, 2016). However, there is still lack of information about tick biology at the cellular and molecular levels, creating an obstacle for this selection.

In the era of next generation sequencing, with the price for massive parallel sequencing technologies decreasing, ticks are still one the more understudied taxons (Gibson, Smith, Fuqua, Clay & Colbourne 2013). The first tick genome fully sequenced was obtained only in 2016 for *Ixodes scapularis* (Gulia-Nuss et al., 2016), followed by a draft assembly for *Rhipicephalus microplus* in 2017 (Barrero et al., 2017), which proved challenging by their size and highly repetitive regions. Apart from these, most of the sequences present in the databases come from RNA-sequencing projects (Antunes et al., 2012; Bissinger et al., 2011; de Marco et al., 2017; Francischetti, Anderson, Manoukis, Pham & Ribeiro, 2011; Heekin et al., 2013; Kotsyfakis, Kopáček, Franta, Pedra & Ribeiro, 2015; Schwarz et al., 2013; Sonenshine et al., 2011), that provide invaluable information for evolutionary analysis, quantitative and differential gene expression, between others (reviewed by Oppenheim, Baker, Simon & DeSalle, 2015). Still, many tick of the genus *Rhipicephalus*, with high veterinary importance, do not have their genome available, slowing down the research for candidate antigens for vaccine development.

In this study, several genes from the folate-related pathways were selected for identification in *Rhipicephalus annulatus* ticks and subsequently analysed for differential expression between uninfected and *Babesia bigemina* infected ticks. Those genes were also analysed in other *Rhipicephalus* ticks, such as *Rhipicephalus bursa* and *Rhipicephalus sanguineus*, uninfected and infected with *Babesia ovis* and *Ehrlichia canis*, respectively, and in the *I. scapularis* tick cell line (IDE8), uninfected and *E. canis* infected. Differential expressed genes were selected for *in vitro* silencing by RNA interference to understand their function and impact in the tick and on the pathogen.

1.1. Ticks species and identification

Ticks can be classified in three families, Ixodidae (hard ticks), Argasidae (soft ticks) and Nutallielidae (including only one specie). Evidence of the existence of ticks can

be traced to the late Palaeozoic era (ca. 300 mya) up to the early Mesozoic era (ca. 200 mya) (Wilde, 1978). More than 850 species of ticks have been reported worldwide being recognized as important vectors of human and animals diseases and for their ability to transmit the widest diversity of pathogens from all arthropod groups (Nava, Guglielmone & Mangold, 2009).

Tick species were originally defined considering their morphology and ecological characteristics (which implied a high level of entomological expertise), but these techniques failed when taxa was too morphologically similar, in non-perfect conditions (damaged specimens) or when in non-described life stages (Nava et al., 2009). With the advances in the sequencing technologies and molecular techniques based on DNA sequence analysis, it was possible to apply an alternative approach. As such, several specimens classifications were significantly modified when molecular data was considered leading to adjustments in the nomenclature (Nava et al., 2009). For example, the alteration of the genus *Boophilus* into a subgenus of the genus *Rhipicephalus* (Murrell & Barker, 2003). Cytochrome c oxidase subunit 1 (CO1) gene is commonly used for species identification, but conclusions should be drawn with care (Sonenshine & Roe, 2014). Other molecular markers have also been used, such as internal transcribed spacer 1 and 2, 18S and 16S ribosomal DNA, but they fail to be universally useful for arthropod species identification and a combination of at least two markers is advised when CO1 leads to an unreliable result (Lv et al., 2014). With molecular identification of tick species becoming a more common procedure it is imperative to optimize the methodology and improve the databases (Diarra et al., 2017; Yssouf, Almeras, Raoult & Parola, 2016).

1.1.1. *Rhipicephalus* ticks

The genus *Rhipicephalus* belongs to the Ixodidae family (which also includes the genus *Ixodes*), subfamily Rhipicephalinae (Fig. 1), is comprised of 82 species (Guglielmone et al., 2010), several of them with high economical and veterinary impact.

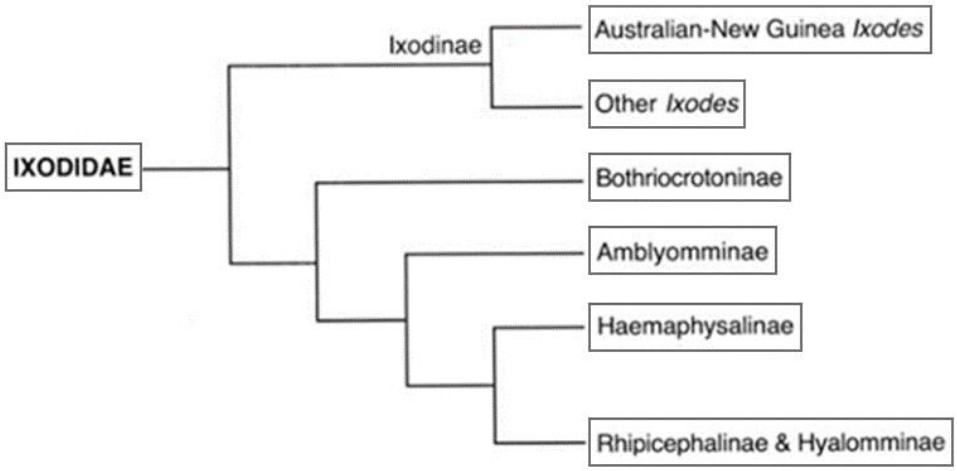


Figure 1 – Proposed phylogeny of the subfamilies of Ixodidae ticks. Adapted from "Systematics and Evolution of Ticks with a List of Valid Genus and Species Names" by S. C. Barker and A. Murrell, 2004, Parasitology, 129 Suppl, S15-36.

Among these, *R. sanguineus* (Fig. 2, A), also known as brown dog tick, is currently the most widespread tick in the world, thriving both in urban and rural areas, infesting dogs and transmitting several pathogens, such as *Coxiella burnetii*, *E. canis*, *Rickettsia conorii* and *Rickettsia rickettsia* (Dantas-Torres, 2010).

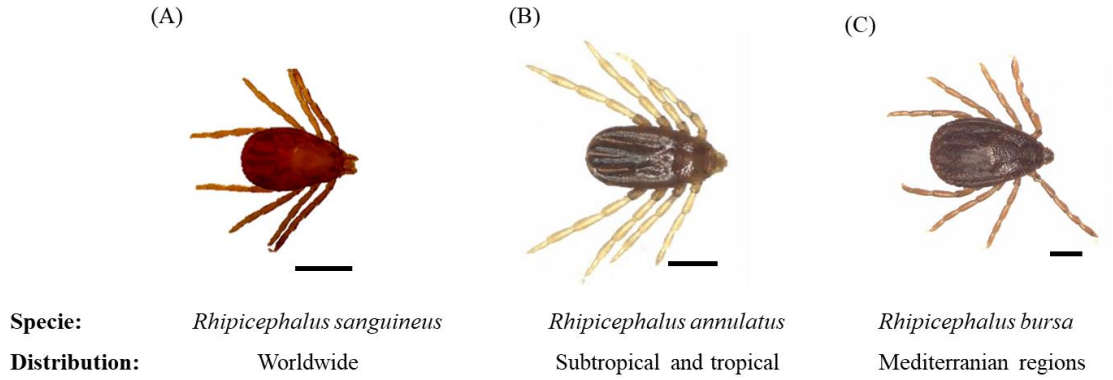


Figure 2 – *Rhipicephalus* spp. female adult ticks, and corresponding geographical distributions (bar = 1 mm). (A) *R. sanguineus*, adapted from "Biology and Ecology of the Brown Dog Tick", *Rhipicephalus sanguineus*" by Filipe Dantas-Torres, 2010, Parasites & Vectors, 3, 26. (B) *R. annulatus* and (C) *R. bursa* adapted from Bristol University Tick ID by Richard Wall retrieved from http://www.bristoluniversitytickid.uk.

R. annulatus (Fig. 2, B) and *R. microplus* are mostly present in subtropical and tropical regions, being a major concern for the livestock industry, either by the damage inflicted by their sheer numbers or by the parasites they transmit (e.g. *B. bigemina* and

Babesia bovis) (Jongejan & Uilenberg, 2004). *R. bursa* (Fig. 2, C) infests primarily ruminants such as sheep and goats and is a vector of *B. ovis*, *Rickettsia* spp. and *Anaplasma* spp. having a wide distribution within the Mediterranean region (Ferrolho et al., 2016; Walker, Keirans & Horak, 2005).

1.1.1.1. Life cycle

Ixodidae tick's life cycle consists of four stages: egg, larva, nymph and adult. Adult female ticks, feed on the blood of the host for several days and once engorged drop off to deposit thousands of eggs, eventually dying. Depending on the specie, ticks may have from one to three hosts if they molt from larvae into nymph and from nymphs into adults on or off the host (Sonenshine & Roe, 2014). However, there is some flexibility in these interactions and under certain conditions ticks can change the number of hosts (Oliver, 1989). The host specificity is also highly dependent on the tick species and the circumstances, with some accepting a wide range of hosts, and others being extremely selective (Jongejan & Uilenberg, 2004). Development periods in all life cycles are strongly impacted by the temperature and humidity (Ogden et al., 2004; Randolph & Storey, 1999).

The majority of ticks from the genus *Rhipicephalus* have three-host life cycles, from which *R. sanguineus* is an example (Fig. 3).

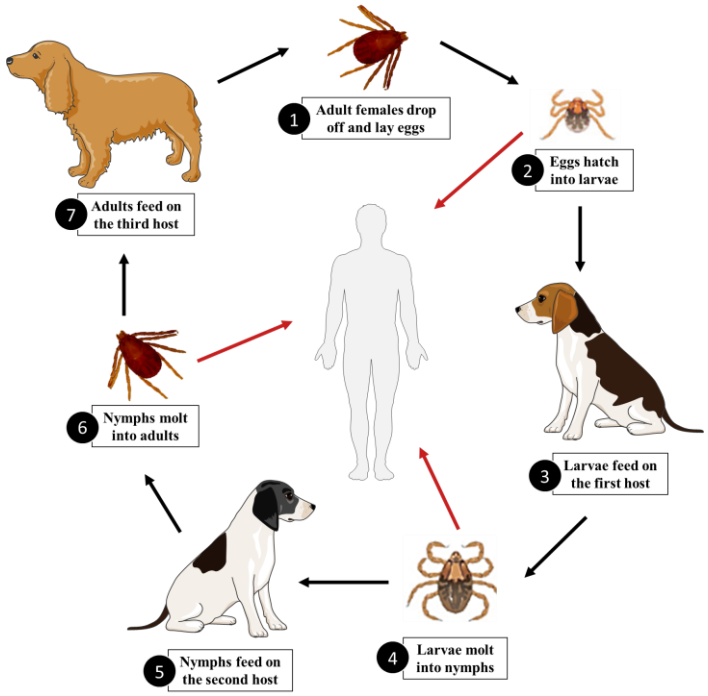


Figure 3 – Three-host ixodid tick life cycle. Representation of the three-host life cycle of the tick *R. sanguineus*, where red lines represent the capacity of these tick to bite and transmit pathogens to humans. Adapted from "Life cycle of *Rhipicephalus sanguineus* and the transmission of *Rickettsia rickettsii* (the causative agent of Rocky Mountain Spotted Fever)" by CDC, May 2017.

After the eggs hatch, larvae search and feed on the first host, dropping off to molt into the nymphal stage. Nymphs quest for the next host, feed and drop off to molt into adults, who will once again search for a host to feed and mate. Engorged females will drop-off and lay the eggs repeating the cycle (Sonenshine & Roe, 2014).

On the other hand, *R. bursa* is a two-host tick (Fig. 4) where usually the larvae molt into nymphs in the same host, dropping off to molt into the adult stage, however it is common for these ticks to recur to the same host for feeding in all life stages (Sonenshine & Roe, 2014; Yeruham, Hadani & Galker, 2000).

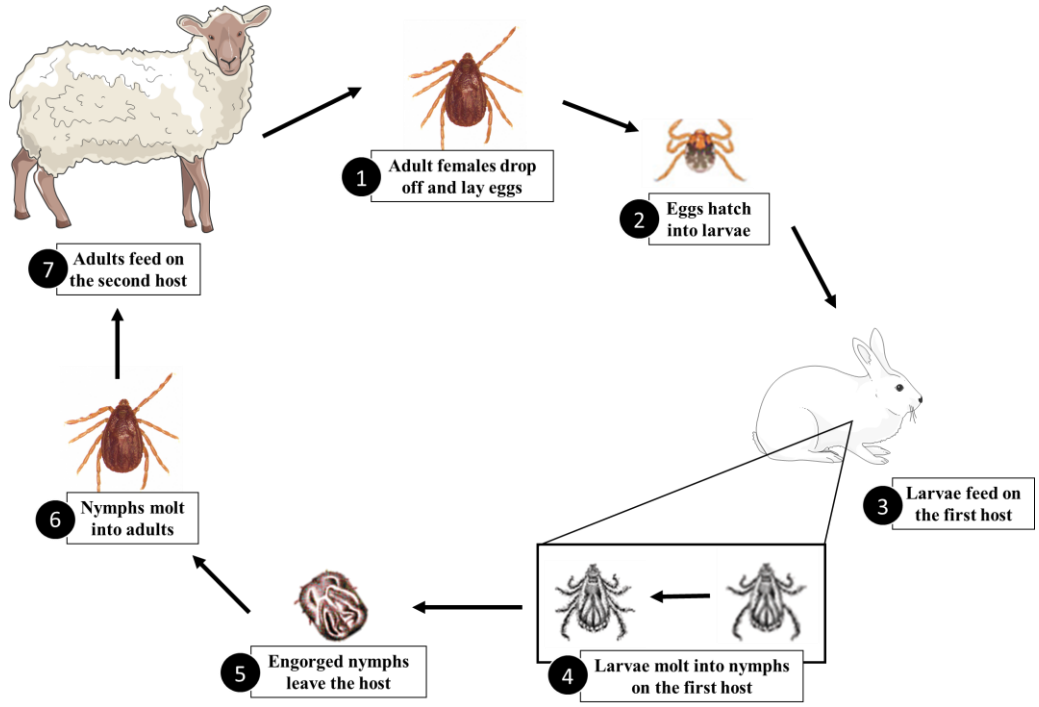


Figure 4 - Two-host ixodid tick life cycle. Representation of the life cycle and hosts of two-host ixodid ticks, such as *R. bursa*. Adapted from "Ticks" by CDC, December 2017.

R. annulatus are characterized for having a single host (Fig. 5), where drop-off only occurs for engorged females (Walker et al., 2003).

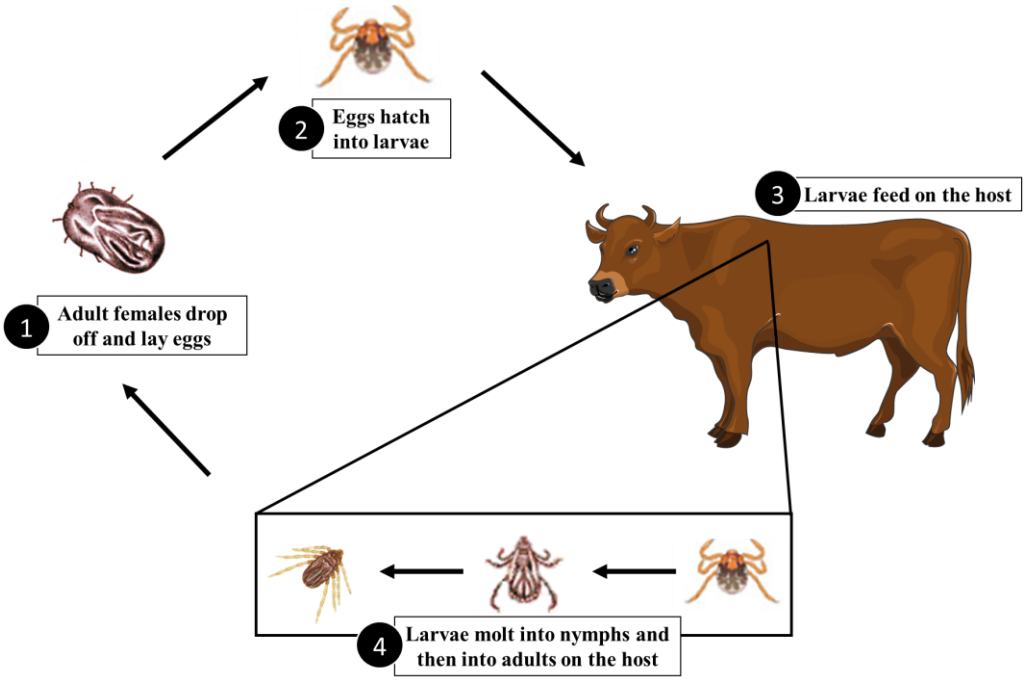


Figure 5 - One-host ixodid tick life cycle. Representation of the life cycle and host of one-host ixodid ticks, such as *R. annulatus*. Adapted from "Ticks" by CDC, December 2017.

1.2. Ticks as vectors

The importance of ticks as vectors has been established for over 100 years (Stockman, 1918) and today these arthropods are well-known to transmit a great diversity of pathogens including virus, bacteria, protozoa and nematodes (Jongejan & Uilenberg, 2004).

The majority of virus transmitted by ticks are RNA viruses with the capacity for transstadial and transovarial infection, meaning they can maintain infection though all the tick life stages and transmit the virus to the next generations (Turell, 1988). These viruses are also able to persist in the tick body until their death increasing the transmission potential (Jones, Nuttall & Davies, 1986). However, tick-virus interactions are still poorly understood and for some viral diseases the tick vector was not yet identified (Mansfield et al., 2017).

New viral diseases associated with tick transmission keep being detected and spreading to previously non-impacted geographical areas. Between these, the Severe Fever with Thrombocytopenia syndrome virus, Heartland virus, Crimean Congo haemorrhagic fever virus, Powassan virus, Deer tick virus, Kyasunar forest disease virus, Alkhurma haemorrhagic fever virus and African swine fever virus were recognized as emerging in the twenty century (Mansfield et al., 2017).

A vast number of pathogenic bacteria are also vectored by ticks. Among these, some are the cause of important human diseases such as the Lyme borreliosis, caused by bacteria of the genus *Borrelia* spp., transmitted by *Ixodes* spp. and Rocky Mountain spotted fever, caused by *R. rickettsi*, transmitted through *Dermancentor variabilis* bite (Parola & Raoult, 2001). In the veterinary field, *R. conorii* (Boutenneuse fever), *Anaplasma* spp. (Anaplasmosis), and *Ehrlichia* spp. (Ehrlichiosis) are highly relevant (Sonenshine & Roe, 2014).

Amongst the protozoa, the apicomplexans *Theileria* spp. and *Babesia* spp. have high veterinary and economic impact, especially in livestock, however they can also cause disease in humans and other animals (Jongejan & Uilenberg, 2004).

1.2.1. Babesiosis

Babesiosis is a TBD, which affects a wide range of vertebrate animals, especially mammals. This illness is caused by the protozoa of the genus *Babesia*, an apicomplexan, that may be compared with *Plasmodium* due to their phylogenetic proximity and several common biological features (Chauvin, Moreau, Bonnet, Plantard & Malandrin, 2009). In some cases, *Plasmodium* spp. is diagnosed instead of *Babesia* spp. in blood smears, especially in malaria endemic regions or in returned travellers from these areas, which leads to an inadequate treatment (Hunfeld, Hildebrandt & Gray, 2008). Even though *Babesia* spp. has been described for its impact on animal health, it has caused increased concern in the last decades as a worldwide emerging zoonosis (Gray, Zintl, Hildebrandt, Hunfeld & Weiss, 2010).

Human babesiosis is most commonly caused by *Babesia microti* and *Babesia divergens* in Europe and USA, however, evidence seems to indicate that several *Babesia* spp. are able to cause human disease around the world (Gray et al., 2010). The parasite species and immunological health of the patient define the clinical picture which ranges from asymptomatic, to severe haemolysis and haemodynamic instability, acute respiratory failure, multi-organ dysfunction and death (Homer et al., 2000; Leiby, 2011; Schnittger, Rodriguez, Florin-Christensen & Morrison, 2012). Transmission mainly occurs through the bite of ticks from the Ixodidae family (Gohil, Herrmann, Günther & Cooke, 2013). Besides the tick bite, there have been reported cases of babesiosis acquired by transfusion of contaminated blood (Homer et al., 2000).

Bovine babesiosis, also known as red water fever is the most impacting TBD of cattle, typically caused by *B. bigemina* and *B. bovis* (Suarez & Noh, 2011). The symptomatology includes hypertension, respiratory stress syndrome, neurological symptoms and severe anaemia that may lead to death (Schnittger et al., 2012). This results, as previously stated, in a high mortality rate, abortions and a decrease in meat and milk production, which greatly affect the livestock industry and livelihood of rural communities, principally in subtropical regions (Sonenshine & Roe, 2014). Amongst the small ruminants (sheep and goats), *B. ovis* is the causative organism of ovine babesiosis and has a great impact on Southern Europe, the Middle East, and some African and Asian countries (Ranjbar-Bahadori, Eckert, Omidian, Shirazi & Shayan, 2012; Yin & Luo, 2007).

1.2.1.1. The genus *Babesia*

The genus *Babesia* is comprised of more than a hundred described species, of which some have the capacity to parasite humans and animals. These apicomplexan protozoans invade and multiply inside the vertebrate host being transmitted by ticks (Schnittger et al., 2012).

Babesia species are usually selective for a tick species inside a determined geographical area but can be quite flexible for the choice of vertebrate host. Therefore, the specificity of the vector will dictate *Babesia* host specificity, since unselective ticks will expose the parasite to a vaster range of vertebrate hosts (Chauvin et al., 2009). This can be seen in species like *B. bovis*, *B. bigemina* and *B. divergens* (low host specificity) which are transmitted by vectors like *R. microplus*, *R. bursa*, *R. annulatus* and *Ixodes ricinus* that feed on very vast range of vertebrate (Chauvin et al., 2009). As such, babesiosis patterns of transmission and geographical distribution are indirectly influenced by all the factors that affect their vectors (Wikel, 2018).

Babesia life cycle involves two hosts: the vertebrate and the tick (Fig. 6).

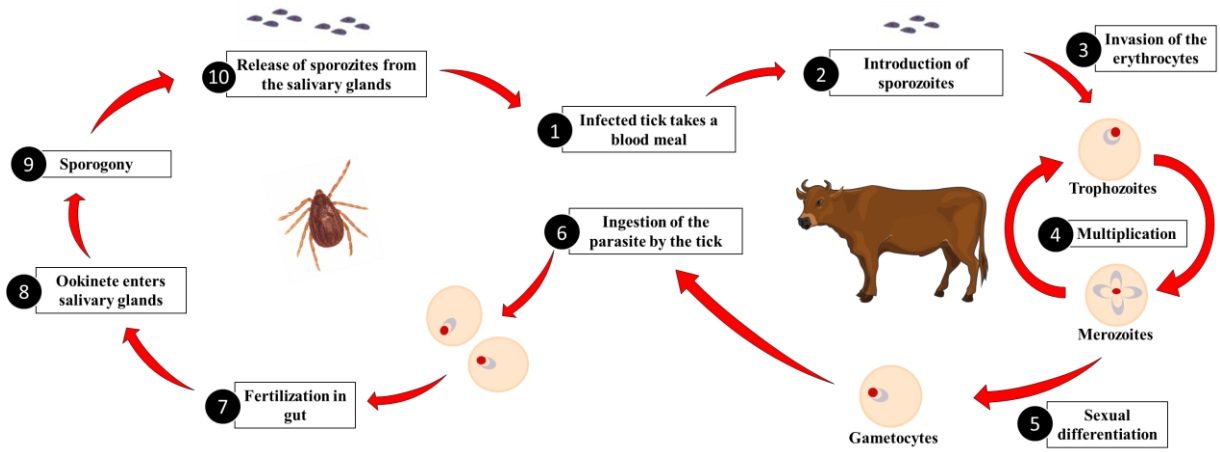


Figure 6 – *Babesia* spp. lifecycle. Representation of the life stages of *Babesia* in the vertebrate host and in the tick vector. Adapted from "Babesiosis – Biology" by CDC, July 2018.

Infection can be acquired during the blood meal of the tick vector, which transmits the sporozoites through the saliva. Those parasites spread into the blood stream and make use of apical complex to invade the erythrocytes, forming a parasitophorous vacuole (Sibley, 2004). Once inside the cells, they can develop into trophozoites and multiply by binary fission into merozoites, leading to the rupture of the red blood cells. The released

merozoites will infect new red blood cells repeating the cycle (Schnittger et al., 2012). Sometimes, four parasites can be present at the same time inside the erythrocyte forming a Maltese cross, a typical structure in *Babesia* sp. infection (Homer et al., 2000). Some of the merozoites will instead develop into gametocytes, the sexual form of the parasite that can initiate infection in the tick during a blood meal on the host (Chauvin et al., 2009).

Once ingested by the tick, the gametocytes will undergo several transformations in the gut, resulting into a zygote that is internalized in the epithelium midgut cells. Here, it will develop into a mobile form, the ookinete, which invades several tissues, such as the ovaries in some species, leading to a transovarial transmission (Chauvin et al., 2009). When ookinetes invade the salivary glands (SGs), sporogony is initiated leading to the formation of new sporozoites that can be transmitted by the vector when the next blood meal occurs (Chauvin et al., 2009; Schnittger et al., 2012).

1.2.2. Ehrlichiosis

Ehrlichiosis is the generic name granted to all infections caused by bacteria from the genus *Ehrlichia*, transmitted through tick bites (Sonenshine & Roe, 2014). In a similar fashion of other Rickettsiosis, it is highly probable to increase in incidence and expand to new geographical areas (Wikel, 2018).

Human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis* has been mostly reported in regions on the USA that coincide with the habitats of the white-tailed deer, a reservoir for the bacteria and host of the vector, the *Amblyomma americanum* tick (Dumler, Madigan, Pusterla & Bakken, 2007). Other bacterial species, such as *Ehrlichia ewingii*, *Ehrlichia muris* and *E. canis* are also able to cause disease in humans. The symptomology is very unspecific and may include fever, headache, myalgias, nausea, arthralgias and malaise (Ganguly & Mukhopadhayay, 2008). Immunocompromised patients may develop a toxic or septic shock-like syndrome, meningitis, respiratory distress syndrome and suffer multiorgan failure which can be life-threatening (Fichtenbaum, Peterson & Weil, 1993; Yachoui, 2013). The non-specificity of the symptoms slows down diagnosis and diminishes the efficiency of the treatment, with statistics indicating that up to 40% of cases require hospitalization and 3% can lead to death (Paddock & Childs, 2003)

Canine ehrlichiosis is a condition with worldwide distribution, most often caused by *E. canis*, transmitted through the bite of the *R. sanguineus* tick (Wikel, 2018). The

symptoms vary with the pathogenicity of the bacterial strain and the immune state of the infected dog. As such, some cases present severe and sometimes life-threatening conditions, while other show no clinical signs of infection, making these individuals optimal reservoirs for dissemination of the disease (Little, 2010). Common symptoms include: acute or chronic fever, lethargy, anorexia, myalgia, splenomegaly ,lymphadenopathy and pancytopenia (Harrus & Waner, 2011; Kelly, 2000). Feline ehrlichiosis has also been described, however seems to be rare in comparison with canine ehrlichiosis (Little, 2010).

1.2.2.1. The genus *Ehrlichia*

Ehrlichia spp. are gram-negative bacteria and the causative agent of human and canine ehrlichiosis. This intracellular pathogen preferably infect monocytes and macrophages, having the capacity to subvert their phagocytic capacity and exploit its cellular systems (Rikihisa, 2006). *E. chaffeensis*, for example, lacks the genes for the biosynthesis of lipopolysaccharide and peptidoglycan, structures normally recognized by the host leukocytes and makes use of a caveolae-mediated endocytosis to avoid fusion with liposomes. These bacteria also can suppress NADPH oxidase, apoptosis, and interferon-γ signalling (Rikihisa, 2006).

The bacteria are acquired by the tick when feeding on an infected host. Once feed, the tick will drop off and after moulting will search for a new host, to whom it may transmit the pathogen. On the vertebrate, these bacteria will replicate inside the monocytes or macrophages. While the pathogen-host interactions have been described (Alves et al., 2014; Wakeel, Zhu, Yu & McBride, 2010) further studies are needed to elucidate the life cycle of *Ehrlichia* in the tick vector.

1.3. Battling against tick and tick-borne diseases

Control and prevention of TBDs is often complex, due to the variety of intervening factors at play, which range from human behaviour, to tick population dynamics and environmental changes (Dantas-Torres, Chomel & Otranto, 2012).

1.3.1. Tick control and transmission blocking alternatives

To overcome the burden associated with ticks and TBDs several strategies have been implement over the years. Vector control is conventionally based on the application of acaricides, however this approach leads to an increase of acaricide resistant ticks and to environment and food product contamination (Abbas, Zaman, Colwell, Gilleard & Iqbal, 2014; Mapholi et al., 2014). Many ticks are specially hard to control since they live in intimate association with their hosts and may occur in areas where acaricide application may not be feasible (Anderson, 2008). Therefore, there is a demand for alternative approaches to tick control.

Other methods, such as interference of tick endosymbionts by the application of entomopathogenic fungi, are quite promising being theoretically safer for the environment and for human health when compared with the use of acaricides, however successful implementation of such strategies was no yet accomplished (Fernandes, Bittencourt & Roberts, 2012). The selection of naturally resistant cattle breeds is also an important strategy in some cases (Mapholi et al., 2014). The release of sterile ticks, in a similar fashion to the sterile insect technique is also an interesting approach (Hilburn, Davey, George & Pound, 1991) but the cost of production of the hybrids associated with the difficulties to obtain public and political support lessen the viability of this approach (Antunes, 2013; Jonsson, 1997).

1.3.1.1. Vaccines

The major alternative for acaricide application so far is the vaccination of the host with tick antigens. The use of *R. microplus* Bm86 gut antigen in commercial vaccines was proved to exert tick control by reduction of infestations by larvae, reducing the number of engorged individuals, their weight and reproductive capacity (de la Fuente et al., 1999; de la Fuente et al., 2007). This discovery marked an important shift into the development of tick vaccines which are often cheaper, environmentally friendlier and less prone to resistance development when compared with acaricides (Mapholi et al., 2014).

In respect to vaccine development several strategies are in place. Since numerous tick species share the same habitat and vertebrate hosts (Estrada-Peña, de la Fuente, Ostfeld & Cabezas-Cruz, 2015), there is an increase interest in the development of

vaccines that may be effective across different vertebrate host species. For example, *R. microplus* recombinant Bm86 antigen proved to be able to control *Hyalomma dromedarii* infestation in cattle and camels (Rodríguez-Valle et al., 2012). On the other hand, there is also an interest in the capacity to offer protection against a vast array of tick species (de la Fuente & Contreras, 2015). The idea of a universal anti-tick vaccine of conserved antigens with the capacity to illicit a protective immune response against several tick species is economically and technically attractive (Parizi et al., 2012). However, so far, few vaccines have proved to be efficient against more than one tick species being extensively review elsewhere (Parizi et al., 2012).

Antigen combinations including several tick antigens, or pathogen-derived antigens is also an alternative approach to increase vaccine efficiency, offering protection to the ectoparasite and also the pathogens by it transmitted (de la Fuente & Contreras, 2015). Further approaches, reside in targeting antigens from the tick-pathogen interface with the potential for a dual control, affecting the vector itself but also the pathogen infection and transmission (Merino et al., 2013).

Selection and validation of proper antigens if often the bottleneck for development of vaccines against ticks and TBDs, since it requires laborious and expensive processes: from identification and evaluation of candidate antigens, analysis of their immunogenic potential to trials in laboratory and wild animals with subsequent cost-efficiency analysis (Rodríguez-Mallon, 2016). The use of technologies, such as RNA interference (RNAi) have been applied for functional analysis in ticks (de la Fuente, Almazán, Blouin, Naranjo & Kocan, 2005) and are especially useful for the characterization of proteins in the tick-pathogen interface and screening of targets for vaccine development (de la Fuente, Kocan, Almazán & Blouin, 2007).

1.4. RNA interference

The RNAi is a natural occurring biological phenomena that defends the organism from foreign genetic material and at the same time has a role in endogenous gene regulation (Mello & Conte, 2004). In a simplified way: the trigger dsRNA is cleaved in to short double stranded molecules, the short-interfering RNA (siRNA). This reaction is catalysed by an enzymatic complex known as Dicer. The siRNAs are then incorporated into the RNA-induced silencing complex (RISC), losing its double stranding conformation

in the process. Once this complex is assembled, the single strand RNA sequence incorporated can, by sequence complementarity, hybridize with the target mRNA that will then be degraded by the enzymatic activity of Argonaut (enzyme included in RISC) (Agrawal et al., 2003; Mello & Conte, 2004). This process can also be initialized by other molecules other than dsRNA, such as aberrant mRNAs or by experimentally provided short-hairpin (shRNA) and pre-micro RNA (pre-miRNA) (Mello & Conte, 2004).

The exploitation of this method allows for specific and intense gene knockdown by translational repression and is a powerful tool for genetic and functional studies, especially in species where other knockdown techniques are not possible or inadequate (Kolev, Tschudi & Ullu, 2011). This mechanism seems to be quite conserved in eukaryotes and have been reported for plants, protozoa, flies, insects, mice and human cell lines (Agrawal et al., 2003). And even prokaryotes, seem to have a RNA-based translation repression mechanism with some similarities to RNAi (van der Oost & Brouns, 2009).

RNAi is now commonly used for gene silencing in ticks allowing functional characterization of genes even though little is known about the mechanism in this particular organism (de la Fuente et al., 2005). Still, studies of this process in other species allow for deductions to be made even if the proteins involved are not yet identified.

RNAi is a powerful tool for antigen screening in the tick-pathogen interface. It allows to minimize the use of laboratory animals when applied *in vitro* and can be used for preliminary characterization of the targets and antigens combinations before preforming more expensive trials (de la Fuente et al., 2007).

1.5. Folate related pathways

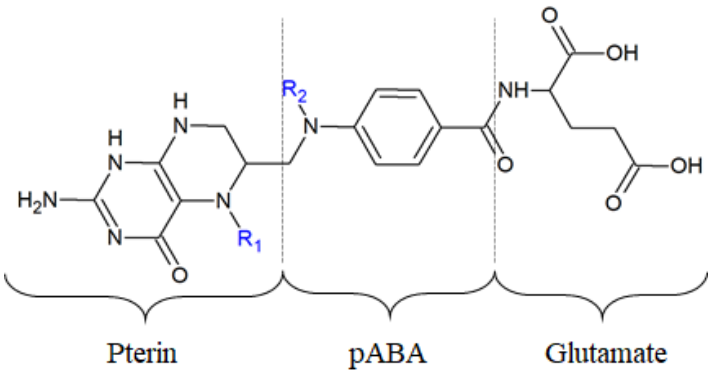
The folate cofactor and derivative compounds have been known for their role in multiple physiological processes within eukaryotic cells, including biosynthesis of purines and thymidine, amino acid homeostasis and redox defences (Ducker & Rabinowitz, 2017).

The broad impact in cell growth and normal development of the organisms, exerted by folate dependent enzymes makes them interesting to study in the context of target selection in vaccine development. Depending on their roles, they may be subdivided in two separate pathways: one-carbon pool by folate and folate biosynthesis. Several enzymes involved in the folate biosynthesis pathway are pivotal in other metabolic processes having implications in the organism survival. While these pathways are well described in

mammals and especially in humans, very little is known about their roles in arthropods and even less so in ticks.

1.5.1. Folate and one carbon-metabolism

Folate is a generic term given to compounds from the vitamin B9 family, essential cofactors for the one carbon metabolism and as such, for the survival and development of the organisms. Folates are heterocyclic compounds, formed by a pterin ring conjugated with para-aminobenzoic acid (pABA) and at least one glutamate residue (Fig. 7).



Compound	R_1	R_2
THF	H	H
DHF	-	H
5-methylTHF	CH3	H
5-formylTHF	CHO	H
10-formylTHF	H	CHO
5,10-methyleneTHF	-CH2-	
5,10-methenylTHF	-CH-	

Figure 7 - Chemical structure of folates. Representation of the common chemical structure of folate compounds, composed by a pterin ring conjugated with para-aminobenzoic acid (pABA) and a glutamate residue. Group substitutions (R_1 and R_2) for each compound are represented in the table. THT, tetrahydrofolate; DHF, dihydrofolate.

The seven most biologically active compounds are: dihydrofolate (DHF), tetrahydrofolate (THF), 5-formyTHF, 10-formylTHF, 5,10-methenylTHF, 5,10-methylene tetrahydrofolate and 5-methylTHF.

The one carbon-metabolism (Fig. 8) is a universal metabolic pathway where folate dependent enzymes primary function is to accept or donate one carbon units: formyl (-CHO), methylene (-CH_2-) and methyl (-CH_3).

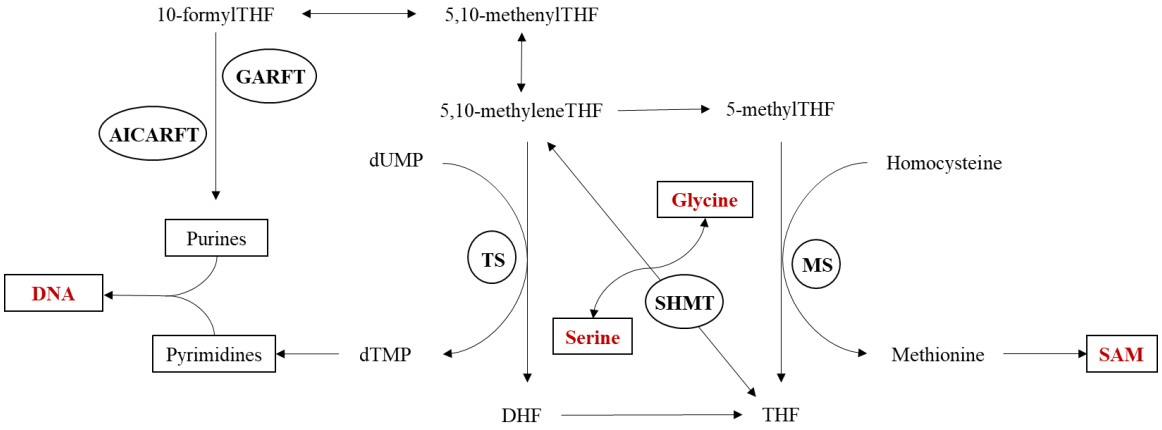


Figure 8 - Simplified overview of the one-carbon metabolism. Interactions between folate compounds and their role in the remethylation of homocysteine, important for production of the cofactor s-adenosylmethionine (SAM) and biosynthesis of purines, pyrimidines, serine and glycine. Relevant enzymes are indicated: thymidylate synthase (TS); serine hydroxymethyltransferase (SHMT); methionine synthase (MS); phosphoribosylglycinamide formyltransferase (GARFT) phosphoribosylaminoimidazolecarboxamide formyltransferase (AICARFT).

Enzyme thymidylate synthase (TS) catalyses the 5,10-methyleneTHF depended conversion of dUMP to dTMP, being the only *de novo* source of this nucleotide and therefore indispensable to DNA replication and repair (Fox & Stover, 2008). The interconversion of glycine to serine is also a 5,10-methyleneTHF depended reaction, catalysed by serine hydroxymethyltransferase (SHMT), where reaction direction is often dictated by the lack of one of these two amino acids in the cell (Ducker & Rabinowitz, 2017). Other important reaction includes the methylation of homocysteine by transfer of the methyl group from 5-methylTHF, catalysed by methionine synthase (MS). This is extremely important step, since the formed methionine is the subtract for S-adenosylmethionine synthetase, responsible for the synthesis of s-adenosylmethionine (SAM). SAM is a high-energy molecule and the major methylation cofactor in the cell, donating methyl groups to phospholipids, proteins, hormones and DNA (Scott & Weir, 1998) and therefore being involved in a vast array of biological phenomena like regulation of DNA, gene transcription, protein localization and catabolic processes (Fox & Stover, 2008). Some folate cofactors are also involved in some steps of the *de novo* biosynthesis of purines. The enzymes phosphoribosylaminoimidazolecarboxamide formyltransferase (AICARFT) and phosphoribosylglycinamide formyltransferase (GARFT), which are part of the same multi-functional enzyme in eukaryotic cells, are responsible for the transfer of the formyl group of 10-formylTHF into different precursors in this pathway (Fox & Stover, 2008).

In summary, the folate dependent one-carbon metabolism controls the levels of serine, glycine and methionine; is directly involved in the production of purines and pyrimidines; is responsible for remethylating of homocysteine and influences the synthesis of SAM (Blatch, Meyer & Harrison, 2010; Selhub, 2002). Besides these primary functions, this pathway can also indirectly influence the biosynthesis of glutathione, creatine and heme, NADPH production and redox homeostasis (Ducker & Rabinowitz, 2017). Suppressions in this pathway may block cell proliferation due to the effect on nucleic acid synthesis (Ducker & Rabinowitz, 2017). Alteration in the homocysteine remethylation are linked with redox imbalance and oxidative stress resulting in the formation of peroxides with cytotoxic potential (Škovierová et al., 2016). SAM availability also impacts epigenetics (Škovierová et al., 2016), a mechanism little characterized in ticks.

The one-carbon metabolism has been intensively studied in humans, with disruptions in this pathways being linked with cancer (Newman & Maddocks, 2017), reproductive problems (Steegers-Theunissen, Twigt, Pestinger & Sinclair, 2013) cardiovascular disease and neural tube defects (Ducker & Rabinowitz, 2017). In insects and arthropods however, very little is known. A study in 1967 indicated that dihydrofolate reductase is essential for mosquitoes oogenesis (Akov, 1967) and in 2003 some folate analogues were proved to be toxic to adult buffalo flies (Elvin, Liyou, Pearson, Kemp & Dixon, 2003).

1.5.2. Folate biosynthesis pathway

Since animals do not have all the genes necessary for *de novo* folate biosynthesis they depend on their dietary intake and salvage from other sources to obtain THF. The blood diet of ticks is meagre in B vitamins which are compensated by the symbiotic relationships these arthropods maintain with their gut microbiome, which do have the capacity to produce these compounds, like some *Rickettsia* species (Hunter et al., 2015). Some apicomplexan, like *Toxoplasma gondii* and *Plasmodium falciparum* parasites seem to own the necessary machinery to synthetize folates *de novo* and salvage from the host (Hyde et al., 2008; Massimine et al., 2005). The presence of this pathway explains these parasites susceptibility to antifolate inhibitors, since of these compounds target enzymes that are present in the *Plasmodium* but are lacking on humans (Müller & Hyde, 2013).

The chemical backbone of folate compounds is constituted by a pterin ring, synthetized from GTP, and pABA synthetized from chorismic acid (Shikimate pathway). These molecules are fused together and undergo glutamylation and reduction reactions to yield THF (Gerdes et al., 2012).

One of the initial key steps of this pathway (Fig. 9) is catalysed by GTP cyclohydolase (GCH-I).

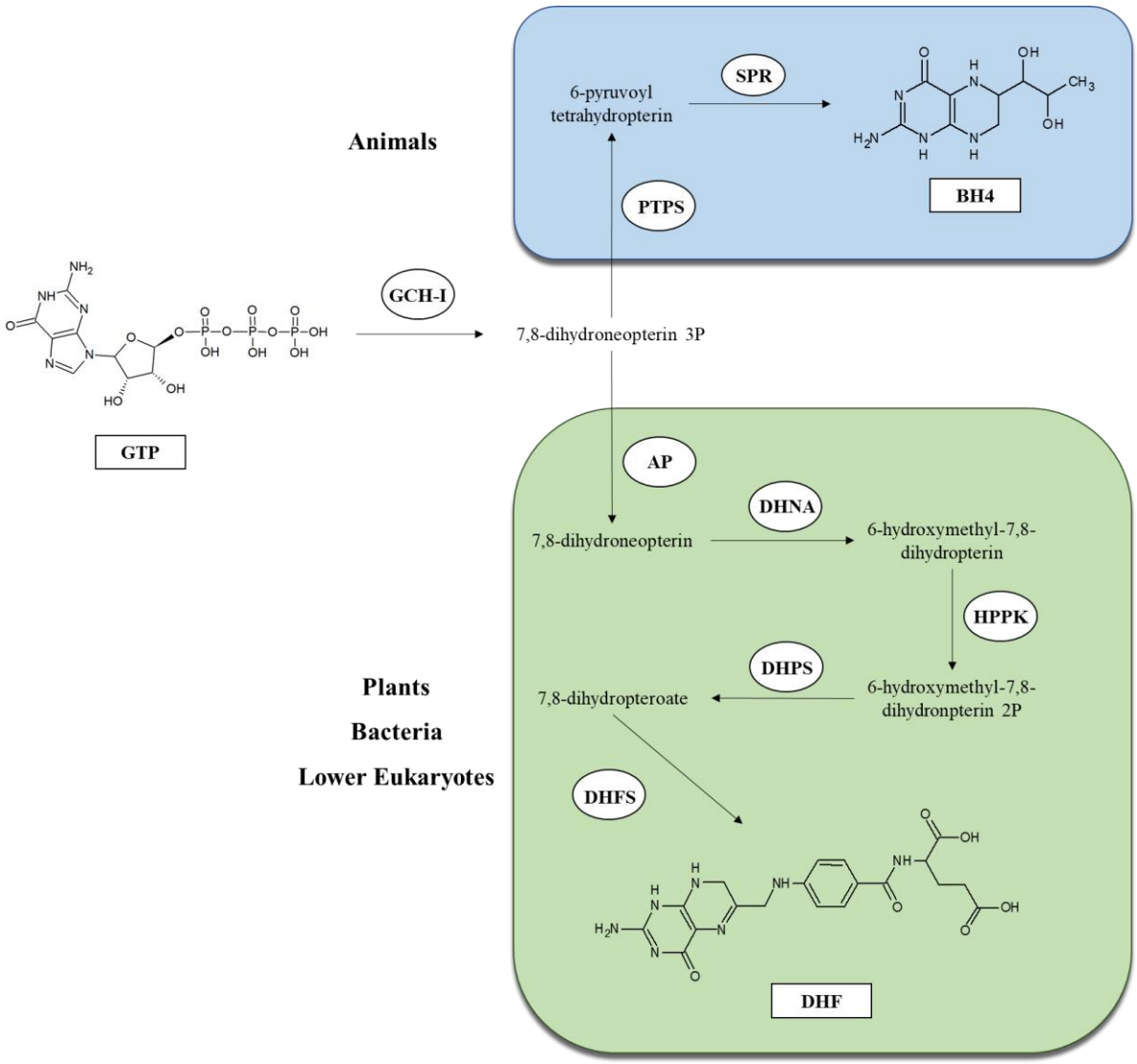


Figure 9 - Simplified overview of the folate biosynthesis pathway. The product 7,8-dihydroneopterin 3P can be used for the synthesis of BH4 in animals (blue box) or folate in plants, bacteria and some lower eukaryotes (green box). Guanosine-5'-triphosphate (GTP); GTP cyclohydrolase I (GCH-I); 6-pyruvoyltetrahydropterin synthase (PTPS); sepiapterin reductase (SPR); tetrahydrobiopterin (BH4); alkaline phosphatase (AP); dihydroneopterin aldolase (DHNA); hydroxymethyldihydropterin pyrophosphokinase (HPPK); dihydropteroate synthase (DHPS); dihydrofolate synthase (DHFS).

The complex sequence of reactions catalysed by GCH-I initiates with the opening and rearrangement of the imidazole ring from GTP and ends with the formation of dihydroneopterin triphosphate (Rebelo et al., 2003). This enzyme is structurally similar between bacteria and animals, although it commits GTP to different pathways: DHF in bacteria and plants and tetrahydrobiopterin (BH4) in animals (Gräwert, Fischer & Bacher, 2013). Biosynthesis of DHF from dihydroneopterin triphosphate includes five important reactions catalysed by alkaline phosphatase (AP), dihydroneopterin aldolase (DHNA), hydroxymethyldihydropterin pyrophosphokinase (HPPK), dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS).

Englobed by the folate biosynthesis pathway umbrella is the biosynthesis pathway of BH4. The dihydroneopterin triphosphate may serve as subtract for DHNA proceeding to THF synthesis or be the subtract for 6-pyruvoyltetrahydropterin (PTPS) synthase committing to the synthesis of BH4. The final step for BH4 biosynthesis is the NADPH-dependent reduction catalysed by sepiapterin reductase (SPR). BH4 is also an essential cofactor for many important enzymes, such as nitric-oxide (NO) synthases and aromatic amino acid hydroxylases, having a key role in several biological processes. In higher organisms it has been linked with formation of amine neurotransmissors like dopamine and serotonin and with cardiovascular and endothelial dysfunction, immune response and pain sensibility (Werner, Blau & Thöny, 2011).

Although deficiencies in BH4 caused by inhibition, are associated with several pathologies in humans (Thöny & Blau, 2006; S. Wang et al., 2008; Werner et al., 2011), there is a lack of information about the biological effects it may have on arthropods, especially on ticks.

Considering the myriad of essential functions attributed to the folate pathway there is an interest to elucidate the role of these enzymes on ticks. Characterization of this pathway may help understand its role on the vector-pathogen interface and disruption may have an adverse effect on tick survival or transmission capacity.

1.6. Aims of this study

Considering the present background and the need for identification of suitable antigens for vaccine development for control of tick and tick-borne diseases, the objectives of this work were:

1. Identification and characterization of the genes of the folate related pathways in *R. annulatus* ticks, followed by differential expression analysis of these genes in uninfected and *B. bigemina* infected ticks.
2. Identification and characterization of the successfully detected genes in *R. bursa, R. sanguineus* and in the IDE8 cell line, followed by differential expression analysis of these genes in uninfected and infected ticks (biological systems: *B. ovis* - *R. bursa; E. canis* - *R. sanguineus* and IDE8 - *E. canis*).
3. Selection of differential expressed genes for *in vitro* functional analysis in the uninfected and *E. canis* infected IDE8 cell line.

To accomplish the first objective, PCR and qPCR methodologies were applied to identify folate related genes in *R. annulatus* ticks and differential expression analysis was conducted by qPCR. The same approach was applied for gene identification in *R. bursa*, *R. sanguineus* and in the IDE8 cell line with differential expression analysis leading to the selection of the *gch-I* gene for functional analysis. *In vitro* gene knockdown by RNAi, of the selected gene allowed to study/suggest its role on invasion and multiplication of the pathogen on tick cells.

2. Material and Methods

Sections 2.1.1.1., 2.1.1.2., 2.1.1.3. and sections 2.1.2., 2.1.3., 2.1.4, 2.1.6., were not performed or partially performed, respectively, in this project. Nonetheless, this contextualization is essential to provide information and background on the origin of the different samples here used.

2.1. Identification of the folate pathway related genes

Linked to the KEGG database (http://www.genome.jp/kegg/pathway.html), arthropod-derived sequences related to folate biosynthesis pathway (map00790) and one carbon pool by folate (map00670) were explored. From UniProt database (http://www.uniprot.org/), the mRNA sequences were retrieved to design primers and the protein sequences subjected to STRING analysis (https://string-db.org/) to evaluate interactions between targets.

The sequences of the primers were designed using Primer3 platform (http://bioinfo.ut.ee/primer3–0.4.0/) and/or PrimerDesign-M (www.hiv.lanl.gov). The primer sequences were evaluated for specificity through the Primer-BLAST tool from NCBI (https://blast.ncbi.nlm.nih.gov) and VectorBase (https://www.vectorbase.org/blast), further oligo analysis was performed with NetPrimer (http://www.premierbiosoft.com/NetPrimer). Primer sequences are listed in Supplementary Table 1.

2.1.1. Ticks and tick-borne pathogens

2.1.1.1. *Rhipicephalus annulatus* and *Babesia bigemina*

R. annulatus ticks were produced at the Kimron Veterinary Institute, Israel. For this purpose, two 6-month-old male Friesian calves were kept in strict tick-free conditions and tested for antibodies against *B. bigemina* by an immunofluorescence assay (Shkap et al., 2005). Non-infected *R. annulatus* ticks were obtain by allowing the ticks to feed on the *Babesia*-free calf, while *B. bigemina*-infected ticks were acquired by feeding in a calf

previously splenectomised and intravenously inoculated with cryopreserved $1x10^6$ *B. bigemina* (Moledet strain) infected erythrocytes (Shkap et al., 2005).

After tick collection, the engorged adult female ticks were maintained at 28 ºC and 80% humidity before shipment to Instituto de Higiene e Medicina Tropical (IHMT) where they were immediately processed.

2.1.1.2. *Rhipicephalus bursa* and *Babesia ovis*

R. bursa ticks were produced at IHMT and kept under laboratory conditions described elsewhere (Antunes et al., 2018). After oviposition each female and egg sample were tested for the presence of pathogens (*Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp.) by conventional PCR (Inokuma et al., 2000; de la Fuente et al., 2003; Aktaş et al., 2005; Harrus et al., 2011). Ticks were allowed to feed in Hyla breed rabbits at Centro de Estudos de Vetores e Doenças Infeciosas, Instituto Nacional de Saúde Doutor Ricardo Jorge (CEVDI/INSA) under suitable conditions. Non-infected *R. bursa* ticks were removed from the ears of the rabbits 10-12 days post attachment while *B. ovis* infected ticks were obtained by inoculation with the parasite in the trochanter-coxae articulation before the attachment and were only collected after drop-off. Ticks were promptly processed.

B. ovis was obtained from a stablished culture based on an adapted protocol from Vega et al. (1985). Culture parasitemia was monitored by blood smears stained with Hemacolor® kit (EMD Millipore, Darmstadt, Germany) and visualized under a 400x original magnification of a Nikon eclipse 80i fluorescence microscope.

2.1.1.3. *Rhipicephalus sanguineus* and *Ehrlichia canis*

R. sanguineus tropical strain ticks were obtained from a pathogen-free colony maintained at the Department of Veterinary Pathology, Faculdade de Ciências Agrárias e Veterinárias – Universidade Estadual Paulista, Jaboticabal, Brazil, under conditions described by Ferrolho et al., 2017. To that end, two male German shepherd dogs about two-month-old were acquired from a certified breeder and vaccinated, dewormed and tested for the presence of *Neospora caninum* (Mineo et al., 2009), *T. gondii* (Domingues et al., 1998), *Babesia vogeli* (André et al., 2010) and *E. canis* (Furuta et al., 2009) by IFA and/or PCR. Non-infected *R. sanguineus* ticks were obtained by allowing the ticks to feed

on an *Ehrlichia* free dog, while *E. canis*-infected ticks fed on a dog previously intravenously inoculated with 5 mL of purified *E. canis*.

E. canis Jaboticabal strain (GenBank no. DQ401044) was purified from a canine monocyte-macrophage cell line DH82 (Wellman, Krakowka, Jacobs & Kociba, 1988) maintained at the Immunoparasitology Laboratory, UNESP, Jaboticabal, São Paulo, as described elsewhere (Aguiar, Saito, Hagiwara, Machado & Labruna, 2007).

2.1.2. Dissection

Ticks were washed in distilled water, 75% (v/v) ethanol and once more in distilled water before dissection in sterile conditions. SGs were dissected with forceps, scalpels and syringes in ice-cold phosphate-buffered saline (PBS) under a stereoscopic microscope at 4x magnification (Motic SMZ-171B, China). Dissected tissues were then stored in RNAlater (Ambion, Austin, TX, USA) at -20ºC.

2.1.3. Tick cell lines

The tick cell line derived from *I. scapularis* embryos (IDE8), obtained from Tick Cell Biobank in the Institute of Infection and Global Health of the University of Liverpool, England under an agreement protocol, was maintained at 32 ºC in sealed flat-sided tubes (Nunc™, Thermo Fisher Scientific, Whaltman, MA, USA) and 25 cm^3 tissue flasks (VWR, Radnor, Pennsylvania, United States,) with L15B medium (Munderloh & Kurtti, 1989). The L15B medium (Gibco, Thermo Fisher Scientific) was supplemented with 10% tryptose phosphate broth (MP Biomedicals, Santa Ana, California, USA), 5% fetal bovine serum (GE Healthcare Europe, Carnaxide, Portugal), 0.1% of bovine lipoprotein (MP Biomedicals), 1% of L-glutamine (200 mM) (Sigma–Aldrich, St. Louis, Missouri, USA), 1% of penicillin (10.000 units)/streptomycin (10.000 µg) (Lonza, Basel, Switzerland) and the pH was adjusted to approximately 6.5. Medium was changed weekly, and subcultures were carried out when a monolayer was reached.

To obtain *E. canis*-infected tick cells, IDE8 were cultured and inoculated with *E. canis* (Spain 105, isolated from a naturally infected dog) at the Tick Cell Biobank. After infection establishment, the culture was shipped to IHMT where was maintained as described previously, except for the absence of antibiotics in the medium. The parasitemia

was analysed every week by microscopic examination of Giemsa-stained cytocentrifuge smears. For this, the cells were resuspended and 100 µL of cell suspension was centrifuged for 5 minutes at 4400 x g (Statspin CytoFuge 2). The cytocentrifuge smears were stained with Hemacolor® Rapid staining kit (EMD Millipore) and examined with a light microscope (Motic, BA210, China), under 1000x magnification with immersion oil. Cell count was performed in a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), and cell viability was estimated with the use of Trypan Blue staining (Sigma–Aldrich).

2.1.4. DNA/RNA extraction

DNA and RNA were extracted from ticks and cell culture using different protocols according previous reports. The number of ticks used for analysis was defined according its availability. For *R. annulatus* samples, total DNA and RNA was extracted from nine uninfected and nine *B. bigemina* infected SGs using the GRS FullSample Purification kit, Grisp Research Solutions, Porto, Portugal). Regarding *R. bursa*, Tri-reagent (Sigma–Aldrich) was used for total RNA extraction of seven individual SGs from *B. ovis* infected and uninfected ticks. For non-infected and *E. canis* infected *R. sanguineus*, the SGs of ten ticks were pooled together for RNA extraction with Tri-reagent. For uninfected IDE8 cells, 8 flasks of $25cm^3$ in monolayer were used while five flat-side tubes with *E. canis* infected IDE8 cells were maintained until an infection rate of about 50% were reached. Total RNA was extracted from approximately $6x10^3$ - $7.3x10^3$ cells/µL for non-infected cells and $6.2x10^2$ - $3.4x10^3$ cells/µL for infected cells using the FullSample Purification Kit.

Quality and integrity of the RNA samples was evaluated with capillary gel electrophoresis in a QIAxcel apparatus (Qiagen, Hilden, Germany), following manufacturer instructions. Nucleic acid concentration for each sample was estimated by ND-1000 Spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific).

2.1.5. cDNA synthesis

The RNA of all the samples was used for the synthesis of cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California, United States), following the manufacturer's protocol performed in a T100 Thermal Cycler (Bio-Rad). The RNA

concentrations for synthesis were normalized between samples of the same group accordingly with the available quantities: 500 ng/µL for *R. annulatus* and IDE8, 250 ng/µL for *R. sanguineus* and 150 ng/µL for *R. bursa.* Several cDNA pools, for all the samples, were also produced for primer optimization and gene screening.

2.1.6. Detection of infection

Validation of *B. bigemina* infection in *R. annulatus* SGs was achieved by Taqman-based qPCR for the amplification of 18S ribosomal DNA (18S rRNA) gene using the primers BiF, BiR and the BiP probe, as described by Kim et al. (2007). The 10 µL reaction included: 5 µL Probe Xpert Fast Probe Mastermix (Grisp), 1 µL of cDNA combined with 0.8 µM of each primer and 0.2 µM of probe. After a first denaturation step at 95 ºC for 10 minutes, followed 45 cycles of denaturation at 95 ºC for 20 seconds and an annealing step at 55 ºC for 1 minute.

Infection of *R. bursa* tick with *B. ovis* was confirmed by the amplification of a 549 bp fragment of *B. ovis* 18S rRNA as described elsewhere (Aktaş et al., 2005).

E. canis infection in IDE8 cells was detected by the amplification of the *dsb* gene by TaqMan qPCR using the primer forward Dsb-321, primer reverse Dsb-671 with a *E. canis* specific probe (5'-AGCTAGTGCTGCTTGGGCAACTTTGAGTGAA-3') like it was proposed by Doyle et al. (2005). The reaction included: 0.8 µM of each primer, 0.2 µM of probe, 5 µL Probe Xpert Fast Probe Mastermix (Grisp) and 1 µL of cDNA.

To validate *E. canis* infection in the pools of *R. sanguineus* SGs, a nested-PCR for the amplification of the 16S rRNA gene was performed, using for the first reaction the primers ECC and ECB which detect all *Ehrlichia* spp., and for the second reaction, the primers ECAN5 and HE3 for *E. canis* specific amplifications (Murphy, Ewing, Whitworth, Fox & Kocan, 1998). The 25 µL reactions included: 12.5 µL of NZYTaq II 2× Green Master Mix, 1 µL of each primer and 5 µL DNA for the first reaction and 1 µL PCR product for the second reaction. The thermocycler conditions were set for both reactions as follow: an initial denaturation for 5 mins at 94ºC; 39 cycles that include: denaturation at 94 ºC for 1 minute, annealing at 60ºC for the first reaction and 55ºC for the second reaction both for 1 minute and extension at 72ºC for one minute; the cycles were followed by a final extension step at 72ºC for 5 minutes. The obtained PCR products were analysed in a 0.5X TBE, 1.2% (w/v) agarose gel with added Xpert Green DNA Stain (Grisp).

2.1.7. Screening of folate related genes with conventional PCR

Traditional PCR was used for screening of the folate related genes in the tick *R. annulatus*. DNA or cDNA were utilized to amplify the fragments of interest, using NZYTaq II 2× Green Master Mix (NZYTech, Campus do Lumiar, Lisboa, Portugal) or KAPA HiFi HotStart PCR Kit, with dNTPs (Kapa Biosystems, Wilmington, Massachusetts, USA) in a T100™ Thermal Cycler (Bio-rad). For the NZYTaq, the 25 µL reaction included: 12.5 µL of NZYTaq II 2× Green Master Mix, primers and DNA/cDNA. For the HotStart Kit, the 25 µL reaction included: 5µL of 5x KAPA Buffer, 0.75 µL of dNTPs, 0.5 µL of KAPA HiFi HotStart DNA Polymerase, primers and DNA/cDNA. The thermocycling steps were programmed as follow: an initial denaturation at 95 ºC for 5 minutes; followed by a denaturation at 98 ºC for 20 seconds, an annealing step with a set temperature or a temperature gradient for 15 seconds followed by an extension step at 72 ºC for 30 seconds, cycling for 35 cycles and a final extension at 72 °C for 10 minutes. A range of primer concentrations and annealing temperatures were tested and are described in Supplementary Table 1.

The obtained PCR products were analysed in a 0.5X TBE, 1.2% (w/v) agarose gel containing Xpert Green DNA Stain (Grisp).

2.1.8. Screening of folate related genes and differential gene expression with qPCR

To complement and validate the screening analysis of the genes and for evaluation of the differential expression of the folate related genes in response to infection in the several biological systems, qPCR was performed. Reactions of 20 or 10 µL were performed in triplicate using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) in a CFX96 Touch Real-time PCR (Bio-Rad), with the following conditions: an initial denaturation step at 95 ºC for 10 minutes; 44 cycles of denaturation at 95 ºC for 15 seconds and annealing at a specific temperature for 30 seconds. A melting curve analysis was also performed (53 - 95 ºC) for evaluation of amplicon quality and reaction specificity. A negative control and a standard curve with 5 or 2-fold serial dilutions of pooled samples were included to validate and determinate the efficiency of each reaction. The information

concerning primer concentrations and annealing temperatures tested are listed in the Supplementary Table 1 and optimized condition are listed in Supplementary Table 2.

For the normalization of the expression profiles, four reference genes were tested: 16S (Zivkovic et al., 2010), *β tubulin*, *β actin* and *elongation factor* (Nijhof, Balk, Postigo & Jongejan, 2009), (Supplementary Table 1). For each biological system, the expression stability value (M-value) of the reference genes was calculated through the *geNorm* algorithm (Vandesompele et al., 2002) incorporated in the CFX Manager™ Software (Bio-Rad). Relative gene expression was assessed on CFX Manager™ Software by the ΔΔCq (Livak & Schmittgen, 2001) and the Pfaff (Pfaffl, 2001) methods. From the normalized expression values, the outliers were singled out by the Tukey method (Tukey, 1977) and Cq-values were compared between conditions by Student's t test. A significant statistical difference was considered when *p*-value was inferior to 0.05 ($p < 0.05$).

2.1.9. DNA purification and sequence analysis

Targets amplified by PCR or qPCR, that exhibited the expected molecular size were purified using NZYGelpure kit (NZYTech) or GRS PCR & Gel Band Purification Kit (Grisp), following manufacturer's protocol, and sequenced by Sanger method (StabVida, Caparica, Portugal). Sequenced fragments were trimmed and analysed by Clustal Omega (Sievers et al., 2011) for homology with the sequences used for the primer design.

2.2. *In vitro* gene knockdown of folate pathway precursors in tick cell culture during *E. canis* infection

2.2.1. dsRNA synthesis

Functional analyse of GTP cyclohydrolase I was performed by RNAi in the IDE8 cell line. As such, double stranded (ds)RNA was synthetized. Firstly, the region of interest was amplified by PCR using the primers specific for GCH-I containing T7 promoter in the 5´ end (Supplementary Table 3). The 25 µL reactions included: 0.02 U/µL of iProof™ High Fidelity DNA Polymerase (Bio-Rad), 1X iProof HF Buffer, 10 mM of dNTP mix, 0.5 µM of each T7 primer, 1 mM of $MgCl_2$ and cDNA from the IDE8 cell line (normalized for 500 ng/µL). Conditions were defined as follow: initial denaturation step at 93ºC for 3 minutes; 35 cycles of denaturation at 98 ºC for 10 seconds, annealing at 60 ºC for 30

seconds followed by an extension step at 72 ºC for 15 seconds; a final extension step was also included at 72ºC for 10 minutes.

Products were examined in a 0.5X TBE, 1.2 % (w/v) agarose gel containing Xpert Green DNA Stain (Grisp), followed by purification with magnetic beads technology (Surf Magnetic Beads, StabVida). The amplified product containing the T7 promoter was used as template for dsRNA synthesis with MEGAscript RNAi Kit (Ambion) following manufacturer's instructions. The purified dsRNA was quantified and analysed in a 0.5X TBE, 1.2 % (w/v) agarose gel with Xpert Green DNA Stain (Grisp).

2.2.2. dsRNA silencing assay in tick cell culture

Inoculation and sample collection were performed at the Tick Cell Biobank in the Institute of Infection and Global Health of the University of Liverpool, England. For the knockdown assay, three experimental groups were defined to study not only the role of the selected gene in tick cells (Group A) but also the impact on the *E. canis* invasion (Group B) and multiplication (Group C) as represented by Figure 10.

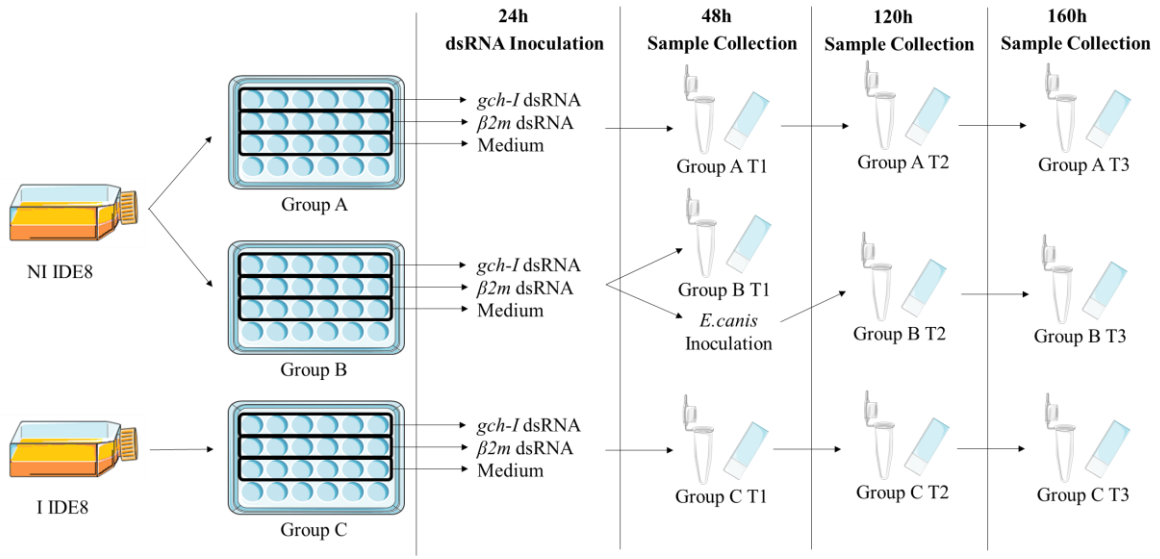


Figure 10 - *In vitro* silencing assay experimental design. Non-infected IDE8 cells and *E. canis* infected cells were distributed in 24 well-plates. After 24 hours, cells were inoculated with *gch-I* or *β2m* dsRNA as represented. Extra medium was added as control of the inoculation procedure. At 48 hours, group B was inoculated with purified *E. canis*. At 48, 120 and 160 hours samples were collected for downstream qPCR analysis (represented by a tube) and for Giemsa-stained cytocentrifuge smears (represented by a slide). (Group A) – Non-infected IDE8 cells; (Group B) – Non-infected IDE8 cells inoculated with *E. canis* at 48 hours; (Group C) – Established *E. canis* infected IDE8 cells; (T1) – 48 hours; (T2) – 120 hours; (T3) – 160 hours.

Group A included non-infected IDE8 cells; Group B comprised IDE8 cells initially non-infected that were inoculated with *E. canis* 48 hours after the beginning of the assay; Group C involved pre-established *E. canis* infected IDE8 cells. First, non-infected and *E. canis* infected cells were maintained in 25 cm^3 flasks. Then, the cells were distributed in 24 well plates to achieve a concentration of $4.16x10^5$ cells/mL in each well. On the next day, cells were inoculated with dsRNA for an approximated concentration of $5x10^{10}$ molecules/µL. Two control groups were included*: β-2 microglobulin* (*β2m*) dsRNA (as non-related dsRNA control) and addition of medium (as a control of the inoculation). At 48 hours, Group B was inoculated with 100 µL of purified *E. canis* from a parasitemia of 80%. For each condition and time point, five pseudo-replicates were collected to evaluate knockdown efficiency and infection rate by qPCR. One well was used for the preparation of Giemsa-stained cytocentrifuge smears for morphological analyse. Three time points were evaluated: 48 (T1), 120 (T2) and 168 hours (T3).

2.2.3. Gene knockdown assessment

All collected samples were centrifuged at 200 x g for 5 minutes and the supernatant was discarded before freezing at -20ºC for subsequent shipment to IHMT and processing. Total DNA and RNA extraction was performed with Tri-reagent (Sigma–Aldrich) as described previously. DNA quantification was performed thought spectrophotometric analyse (NanoDrop ND1000, Thermo Fisher Scientific) and absolute quantification of *E. canis* was attempted by amplification of the *dsb* gene, as described previously. DNA quality was checked by attempting to amplify the 16S tick housekeeping gene.

As mentioned before, quality and integrity of the RNA samples were evaluated, and RNA was normalized for a concentration of 250 ng/µL to serve as template for cDNA synthesis with iScript™ cDNA Synthesis Kit (Bio-Rad), and subsequent qPCR analysis.

Several reference genes were tested - *β-tubulin*, *β-actin*, *16s*, *r13a* and *rpl4* – and the M-value was evaluated through the geNorm algorithm. Relative gene expression was calculated, and outliers excluded as described in section 2.1.8. Assessment of the gene knockdown was performed by comparison of gene expression between the samples exposed or not to *gch-I* dsRNA and *β2m* dsRNA for all conditions.

The effect on cell viability and *E. canis* morphology was examined through the Giemsa-stained cytocentrifuge smears. Infection rate was evaluated thought qPCR by

amplification of *dsb* gene. M-values of three reference genes were calculated *16s*, *r13a* and *β-actin*. The inoculum of *E. canis* was defined as the calibrator sample with relative expression level of 1. Relative normalized gene expression was assessed on CFX Manager™ Software by the ΔΔCq and the Pfaff methods, outliers were excluded by the Tukey method, as described previously in section 2.1.8. Statistical analysis was executed on GraphPad Prim software (version 7.05 for Windows), the Saphiro-Wilk test (Shapiro & Wilk, 1965) supported the normal distribution of the samples, however the Levene test (Olkin, 1960) proved lack of equality of variances which coupled with the uneven sample sizes between groups supported the selection of a non-parametric test. Cq-values were compared between conditions by Mann-Whitney test. A significant statistical difference was considered when *p*-value was inferior to 0.05 ($p < 0.05$).

3. Results and Discussion

3.1. *In silico* analysis of folate-related genes

Folate related compounds and enzymes are involved in several essential biological processes having a broad impact in cell growth and normal development of the organisms. Some of these are universal, being found in arthropods, bacteria and apicomplexan parasites. Interestingly, several genes from the "one carbon pool by folate" (KEGG pathway: map00670, Fig. 11) and "folate biosynthetic pathway" (KEGG pathway: map00790, Fig. 12) were represented in previously obtained RNA-seq data from uninfected and *B. bigemina* infected *R. annulatus* ticks (unpublished data).

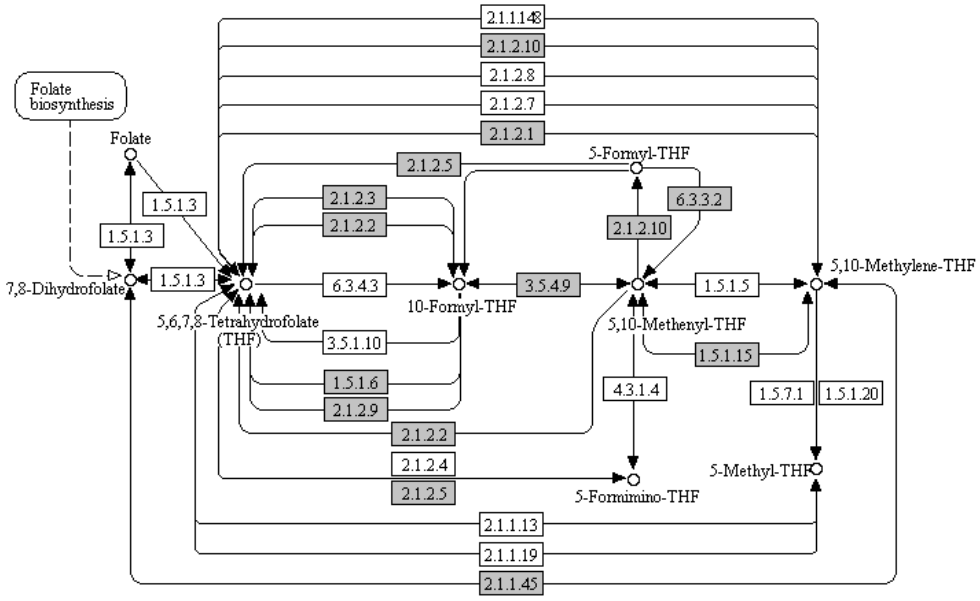


Figure 11 - One carbon pool by folate pathway. Adapted from map 00670 of KEGG database (http://www.genome.jp/kegg/pathway.html). Selected enzymes are shaded grey. Numbers indicate enzyme commission numbers, balls represent compounds, arrows indicate the direction of the reaction and doted lines indicate connections with other pathways.

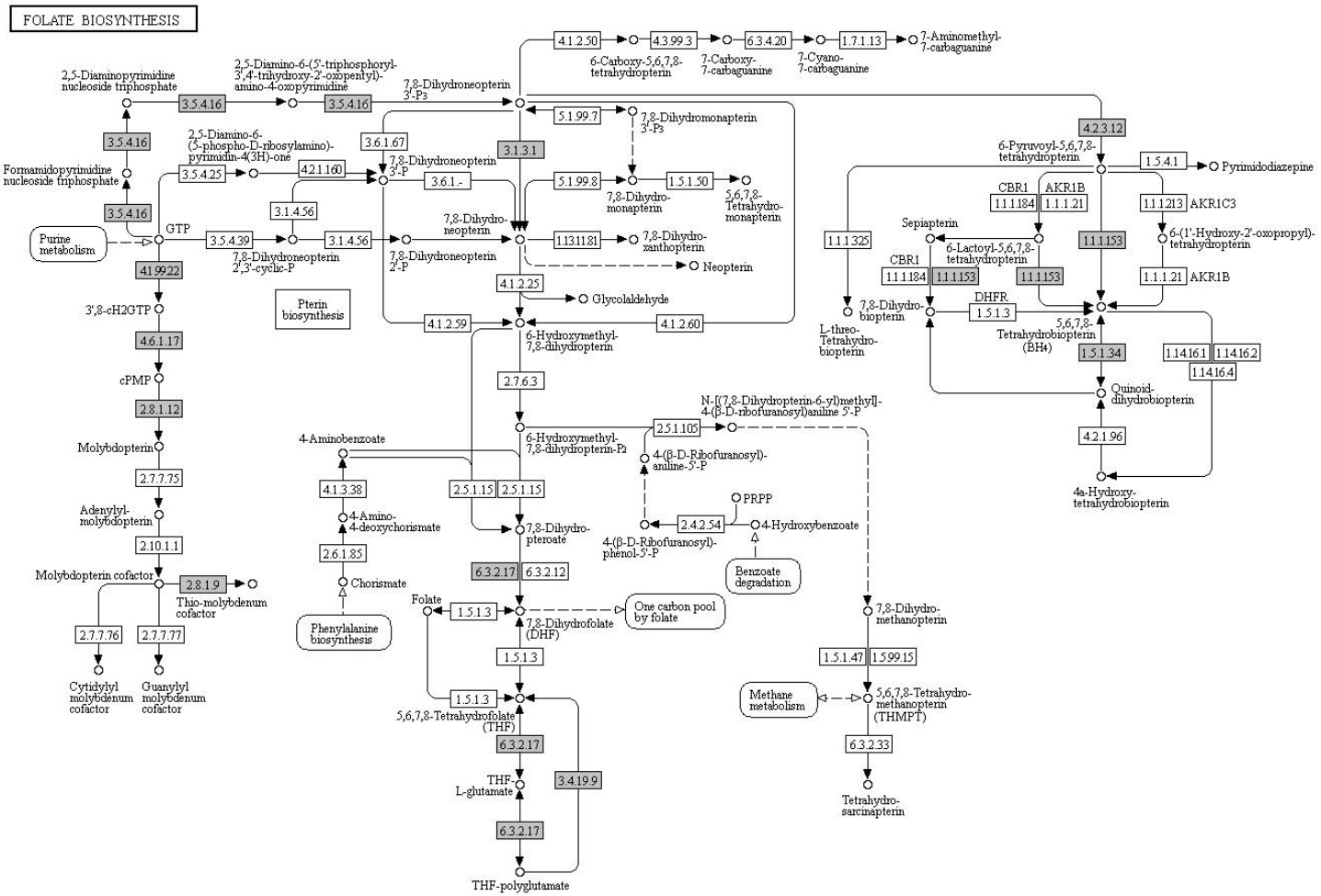


Figure 12 - Folate biosynthesis pathway, adapted from map 00790 of KEGG database (http://www.genome.jp/kegg/pathway.html). Selected enzymes are shaded grey. Numbers indicate enzyme commission numbers, balls represent compounds arrows indicate the direction of the reaction and doted lines indicate connections with other pathways.

As such, several genes from these pathways were selected for evaluation of expression profiles in infection.

Based on the information from KEGG maps 00670 (Fig. 11) and 00970 (Fig. 12) from *I. scapularis*, key enzymes from the pathway were individually subjected to STRING analysis to predict protein-protein interactions (Fig. 13).

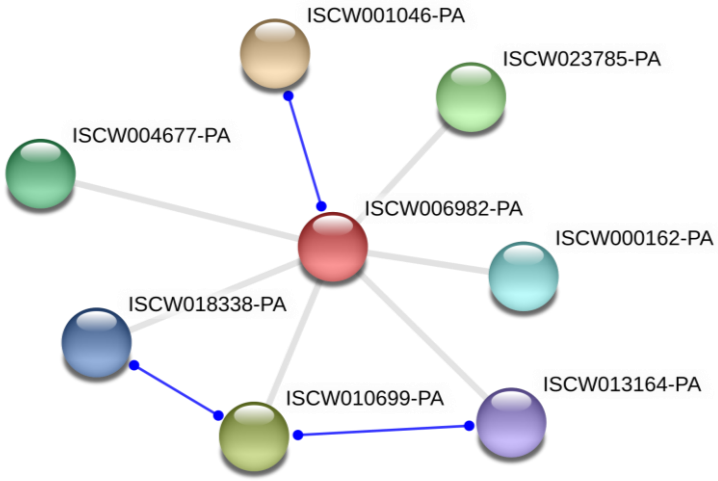


Figure 13 - STRING analysis of GCH-I. Network of predicted associations for GCH-I (ISCW006982). The nodes represent proteins AP (ISCW023785, ISCW004677, ISCW000162), MCBPP1 (ISCW018338, ISCW010699), MCBPP2 (ISCW013164) and GCHFR (ISCW001046-PA). The lines represent predicted protein-protein interactions: grey line – reaction; blue line – binding. The round end of the lines represents an unspecified action effect.

Proteins presented by the analysis with an interaction score of above 0.9 were included in the study. Enzymes showing an apparent non-redundant role in the pathways and related enzymes found by the STRING analysis were selected for characterization in the *R. annulatus – B. bigemina* biological system (Table 1).

Table 1 - List of selected enzymes. Enzymes for characterization in the biological system *Rhipicephalus annulatus – Babesia bigemina* with corresponding enzyme commission numbers (E.C.) and UniProt ID entries retrieved for primer design.

Enzyme Name	E.C.	UniProt ID
GTP cyclohydrolase I (GCH-I)	3.5.4.16	B7PWM4
Alkaline phosphatase (AP)	3.1.3.1	B7QGN3 B7PF40 B7P0L3
Thymidylate synthase (TS)	2.1.1.45	B7P7E2 L7M0X3
Folylpolyglutamate synthase (FPGS)	6.3.2.17	B7PVJ7

Gamma-glutamyl hydrolase (GH)	3.4.19.9	B7PQJ1
6-pyruvoyltetrahydropterin synthase (PTPS)	4.2.3.12	G3MHQ8
Sepiapterin reductase (SPR)	1.1.1.153	B7QMY1 B7QGH5 B7PCLO
Dihydropteridine reductase (DHPR)	1.5.1.34	B7QAP3
Molybdenum cofactor biosynthesis pathway protein 1 (MCBPP1)	4.1.99.22 4.6.1.17	B7PFI1 B7Q4E2
Molybdopterin synthase (MPTS)	2.8.1.12	B7Q3T4
Molybdenum cofactor sulfurtransferase (MCS)	2.8.1.9	Q9VRAZ X2JFY7
Molybdenum cofactor biosynthesis pathway protein 2 (MCBPP2)	-	B7QGD6 B7PDW8
Molybdopterin synthase large subunit (MSLS)	-	B7PDW7
Aminomethyltransferase (AMT)	2.1.2.10	B7P6X5
Serine hydroxymethyltransferase (SHMT)	2.1.2.1	B7PG87
Glutamate formiminotransferase (FTCD)	2.1.2.5	B7Q6E5
Phosphoribosylaminoimidazolecarboxamide formyltransferase (AICARFT)	2.1.2.3	B7PV68
Phosphoribosylglycinamide formyltransferase (GARFT)	2.1.2.2	B7P986
Aldehyde dehydrogenase (ALDH)	1.5.1.6	B7Q8K1
Methionyl-trna formyltransferase (MFT)	2.1.2.9	B7QKW8
Methenyltetrahydrofolate cyclohydrolase (MTHFC)	3.5.4.9	B7QFP4
5-formyltetrahydrofolate cyclo-ligase (MTHFS)	6.3.3.2	B7Q8Q2

Some of the proteins retrieved from the STRING analysis, such as Molybdopterin synthase large subunit (MSLS) and Molybdenum cofactor biosynthesis pathway protein 2 (MCBPP2) were not associated in the databases with an enzyme commission number. Multi-protein STRING analysis of the selected enzymes confirmed their interactions and the intricate interplay between them, assuming a minimum interaction score of 0.4 (Fig. 14).

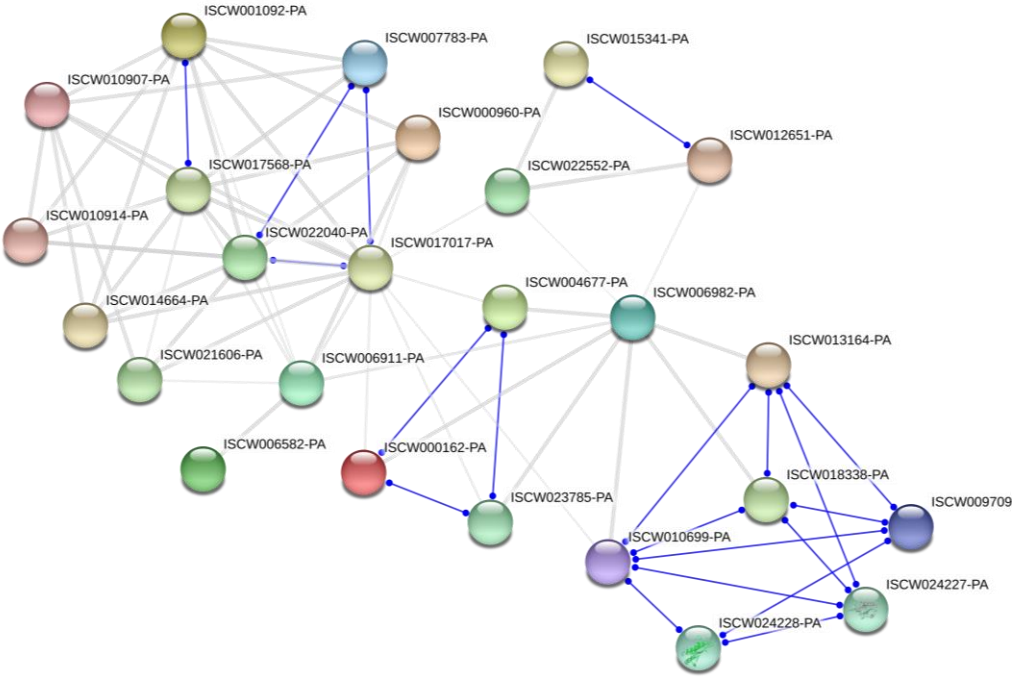


Figure 14 - STRING analysis of selected targets. The nodes represent proteins: GCH-I (ISCW006982-PA), AP (ISCW000162-PA, ISCW004677-PA, ISCW023785-PA), TS (ISCW000960-PA), FPGS (ISCW006911-PA), GH (ISCW006582-RA), SPR (ISCW012651-RA, ISCW015341-RA, DHPR (ISCW022552-PA), MCBPP1 (ISCW010699-RA, ISCW018338-RA), MPTS (ISCW009709-RA), MCBPP2 (ISCW013164-RA, ISCW024228-RA), MSLS (ISCW024227-RA), AMT (ISCW001092-PA, SHMT (ISCW017568-PA), FTCD (ISCW010907-PA), AICARFT (ISCW007783-PA), GARFT (ISCW017017-PA), ALDH (ISCW010914-RA), MFT (ISCW014664-PA), MTHFC (ISCW022040-RA) and MTHFS (ISCW021606-PA). The lines represent predicted protein-protein interactions: grey line – reaction; blue line – binding. The round end of the lines represents an unspecified action effect.

Stronger confidence is observed within some clusters, namely between those involved in the biosynthesis of the molybdenum cofactor, an essential transition micronutrient (Mendel, 2013) and between GARFT, AICARFT and MTHFC, enzymes involved in the purine biosynthesis.

To proceed with the characterization of those key enzymes, the corresponding mRNA sequences were needed for primer design. However, a limited amount of genomic data from ticks is currently available. So far, only the genome for *I. scapularis* has been fully sequenced (Gulia-Nuss et al., 2016) and, very recently, a draft assembly of the *R. microplus* was reported (Barrero et al., 2017). Regarding *R. annulatus*, it counts only with 96 entries for ESTS in Gene Bank (Antunes et al., 2012), a total of 108 nucleotide sequences deposited, and 54 entries on UniProt, being all unreviewed.

Therefore, the search for folate-related coding sequences from *R. annulatus* proved to be ineffective in these databases. As such, conserved regions found in orthologous

sequences from *I. scapularis* and/or other evolutionary closer organisms (other *Rhipicephalus* spp., *Amblyomma* spp. and even *Drosophila melanogaster*), when available, were used for primer design in this study. When low conservation across species was apparent, degenerated primers were designed that would allow some degree of polymorphism in the sequences (Yoon & Leitner, 2015).

A total of 22 proteins were selected, however for some of these the STRING analysis allowed the retrieval of more than one Uniprot ID entry associated with the same E.C. number. When the associated mRNA sequences showed low similarity, primer pairs where designed for each sequence. All 36 primer pair sequences and correspondent sequence entry codes used for their design are discriminated in Supplementary Table 1. For simplicity, gene names are represented with the same acronyms of their associated proteins.

3.2. PCR and qPCR primer optimization and amplification of folate related genes in *R. annulatus*

An initial screening and optimization attempt for the primer pairs was performed by traditional PCR using *R. annulatus* cDNA. Of the 22 primer pairs tested by this technique, only three genes showed specific amplification – *gch-I*, *ts* and *ptps*. Primer specificity was confirmed by the presence of a single band (with approximately the expected amplicon size) in the electrophoresis gel, as exemplified in Fig 15.

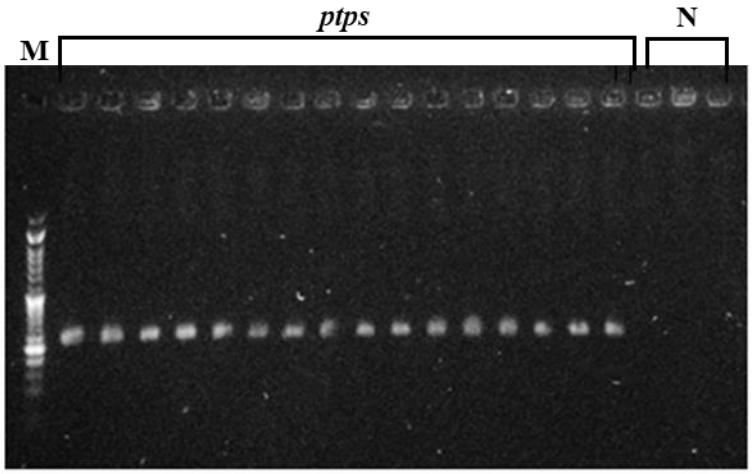


Figure 15 – Amplification of gene *ptps*. Agarose gel electrophoresis of PCR product amplified by primers for gene *ptps* with cDNA pool of *R. annulatus* as template. From left to right: Marker, NZYDNA Ladder VI (M); gradient of temperature from 60 to 55ºC, negative controls (N).

Due to the inability to amplify and optimize the majority of the primer pairs by traditional PCR, an additional optimization attempt was performed by qPCR. The higher sensibility of this method allows the detection of low concentrations of the target gene that could not be detected in an electrophoresis gel. Of the 31 primer pairs tested by this methodology only two more genes showed specific amplification – *shmt* and *acarft*. Primer specificity was ensured by analysis of the melt curves, that presented a single peak (Fig. 16) and by the presence of a single band in the in the electrophoresis gel, as described above.

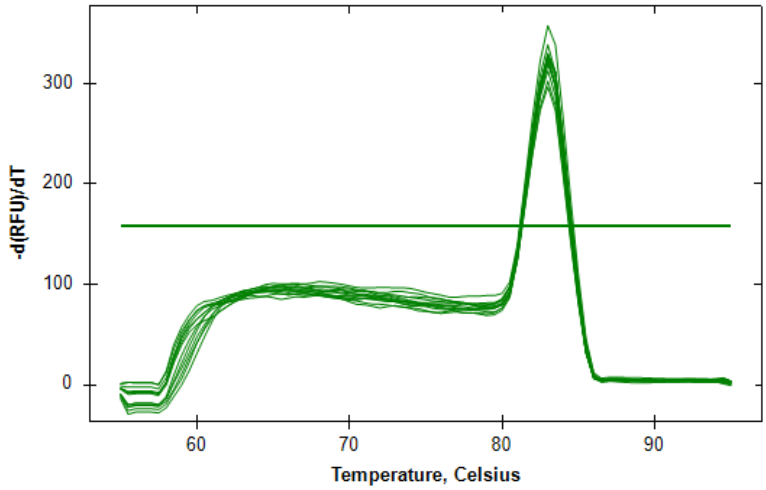


Figure 16 - Melting curve obtained for *shmt*. Vertical axis represents changes in the fluorescence signal over time (-d(RFU)/dT) plotted against the temperature in the x-axis.

All primer pairs that showed non-specific amplification in all the tested conditions were excluded from the following assays.

For validation of primer specificity, both PCR and qPCR products were purified and sent for Sanger sequencing. The obtained sequences showed high identity with the retrieved sequences and as such, were considered to correspond to folate-related genes (Table 2).

Table 2 - Sequence identity of *Rhipicephalus annulatus* amplified genes. Genes with the corresponding retrieved sequences in UniProt database. Identity percentage between sequences was calculated by Clustal Omega.

Gene	Uniprot ID	Specie	Identity (%)
gch-I	B7PWM4	*Ixodes scapularis*	78.30
ts	L7M0X3	*Rhipicephalus pulchellus*	91.57
ptps	G3MHQ8	*Amblyomma maculatum*	84.85
shmt	B7PG87	*Ixodes scapularis*	78.00
aicarft	B7PV68	*Ixodes scapularis*	80.70

As such, it was possible to identify in *R. annulatus* two genes belonging to the folate biosynthesis pathway – *gch-I* and *ptps*- and three genes from the one carbon pool by folate pathway – *ts*, *shmt* and *aicarft.*

The low success for the amplification of the remaining genes could be related to the lower homology between *R. annulatus* and other tick species genes or lower expression of those folate-related genes.

3.3. Differential gene expression in *R. annulatus – B. bigemina*

After optimization was ensured, qPCR was used to analyse differential expression of the selected genes (*gch-I*, *ts*, *ptps*, *shmt* and *aicarft*) in uninfected and *B. bigemina*-infected *R. annulatus* ticks.

In this study, RNA extracted from SGs of eighteen *R. annulatus* ticks was used to produce cDNA, that would work as template for the qPCR reactions. Of these ticks, nine were uninfected and the other nine infected with *B. bigemina*, as confirmed by a qPCR assay using a TaqMan probe for the 18S region of the *B. bigemina* genome (Fig. 17).

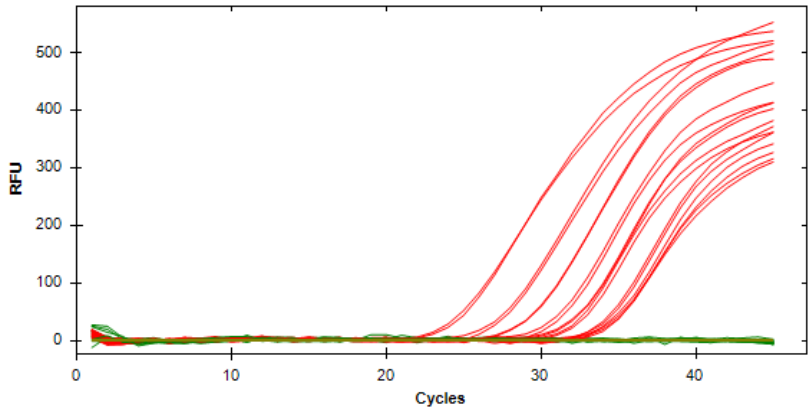


Figure 17 - Amplification chart for *Rhipicephalus annulatus* samples. Fluorescence in the y-axis against the number of reaction cycles in the x-axis. Red lines represent *B. bigemina* infected samples and green lines represent uninfected *R. annulatus* ticks. All infected samples showed an amplification curve while non-infected samples had no amplification.

After confirming infection, data normalization for *R. annulatus* was performed recurring to the genes *β-tubulin, β-actin*, *16s* and *elf*, which served as invariant endogenous controls. For the five genes tested – *gch-I, ts, ptps, shmt* and *aicarft* - statistically significant differential expression ($p < 0.05$) was only obtained for *gch-I*, *ptps* and *shmt*, however, all genes showed an apparent overexpression when the ticks where infected with *B. bigemina* (Fig. 18).

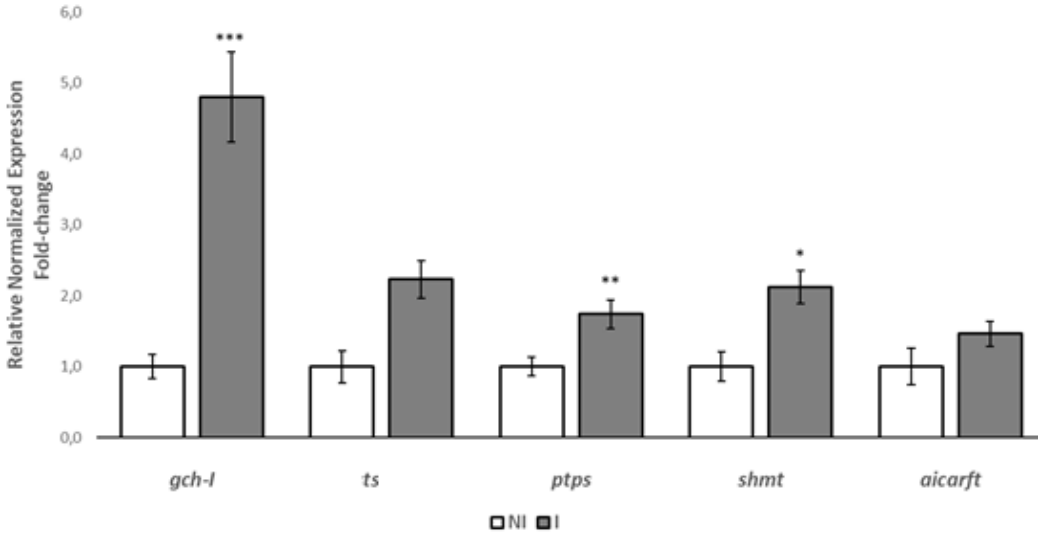


Figure 18 - Differential gene expression in uninfected and *Babesia bigemina* infected *Rhipicephalus annulatus* ticks. Relative normalized expression of *gch-I, st, ptps, shmt* and *aicarft* genes for the infected (I) samples against the uninfected (NI) controls. Significant statistical differences are indicated with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

The normalized data indicated an approximated 5-fold increase in expression for the *gch-I*, 1.74-fold for *ptps* and nearly 2-fold for *shmt.*

The genes, *gch-I* and *ptps* which code for the two first proteins involved in the biosynthesis of BH4 are over expressed in the presence of *B. bigemina*. BH4 is an essential cofactor for production of NO (Sakai, Kaufman & Milstein, 1993), a molecule with antimicrobial activity involved in several innate immunity mechanisms including phagocytosis (Bogdan, Röllinghoff, & Diefenbach, 2000). Therefor up regulation of the enzymes involved in its production suggests that this phenomenon may be a stress response induced by the parasite. *T. gondii* infections in mice have been described to induce production of NO (Dincel & Atmaca, 2015).

Expression of *shmt* is also significantly altered. The SHMT enzyme is responsible for the interconversion of serine and glycine amino acids, also producing 5,10-formylTHF a primary source of activated one carbon units, required for thymidylate biosynthesis and remethylation of homocysteine. A study on female mosquitos, showed that natural regulation of SHMT thought miRNA regulates sugar absorption and blood intake (Liu, Lucas, Roy, Ha & Raikhel, 2014). While overexpression of this enzyme can be associated with increased DNA replication and cellular multiplication, the role of the parasite in this response is difficult to precise.

The showed tendency seems to indicate an impact of the parasite in these folate-related pathways of *R. annulatus*, which can either be resulting of: a response of the tick organism to the presence of the parasite, a response against the parasite, or even a parasite manipulation of the tick resources for its benefit (de la Fuente et al., 2007).

3.4. qPCR primer optimization and amplification of folate related genes in *R. bursa*, *R. sanguineus* and IDE8

To understand if the differential expression observed in the *R. annulatus – B. bigemina* biological system, was a species-specific phenomenon or if these tendencies could also be present in a vaster range of tick–pathogen interplay, an attempt to characterize these six genes in other stablished biological systems such as *R. bursa – B. ovis* and *R. sanguineus – E. canis* was performed. The same analysis was applied for IDE8 cells (*I. scapularis* derived cell line) to comprehend if this *in vitro* system could serve as a first approach in differential expression studies with *Rhipicephalus* ticks and could be used for silencing assays.

From the five genes successfully amplified for *R. annulatus*, only three could be amplified by qPCR in *R. bursa* and *R. sanguineus* - *gch-I, ts* and *ptps*. As such, only these three genes were analysed in the IDE8 cell line. As described above, primer specificity was confirmed by analysis of the melt curves and electrophoresis gel. The purified qPCR products were sent for Sanger sequencing and showed a high identity with the reference sequences (Table 3).

Table 3 - Sequence identity of *Rhipicephalus bursa*, *Rhipicephalus sanguineus* and IDE8 amplified genes. Genes and corresponding retrieved sequences in UniProt database**.** Identity percentage between sequences was calculated by Clustal Omega.

Specie	Gene	Uniprot ID	Species	Identity (%)
R. bursa	*gch-I*	B7PWM4	*Ixodes scapularis*	78.31
	ts	L7M0X3	*Rhipicephalus pulchellus*	93.02
	ptps	G3MHQ8	*Amblyomma maculatum*	79.57
R. sanguineus	*gch-I*	B7PWM4	*Ixodes scapularis*	71.31
	ts	L7M0X3	*Rhipicephalus pulchellus*	84.66
	ptps	G3MHQ8	*Amblyomma maculatum*	80.51
IDE8	*gch-I*	B7PWM4	*Ixodes scapularis*	96.89
	ts	B7P7E2	*Ixodes scapularis*	99.39
	ptps	V5H6X5	*Ixodes ricinus*	96.46

Lack of amplification of the *shmt* and *aicarft* genes in *R. bursa* and *R. sanguineus* indicates low homology for theses sequences between the *Rhipicephalus* genus, since the reaction was successful for *R. annulatus* ticks.

As expected, amplification products obtained for the IDE8 cell line with primers designed with *I. scapularis* (B7PWM4, B7P7E2) or *I. ricinus* (V5H6X5) sequences presented a high sequence identity (> 96 %).

3.5. Differential gene expression in *R. bursa* - *B-ovis*, *R. sanguineus* - *E. canis* and IDE8 – *E. canis*

After primer optimization for *R. bursa*, *R. sanguineus* and IDE8 was accomplished, qPCR was used for analysis of the differential expression of the genes *gch-I, ts and ptps.* RNA was extracted from SGs of 14 *R. bursa* ticks, seven uninfected and seven infected with *B. ovis*. The cDNA synthetized served as template for the qPCR reactions. *Babesia* infection was confirmed by the amplification of a 549 bp fragment of *B. ovis* 18S rRNA. For *R. sanguineus*, RNA was extracted from three pools of ten SGs of uninfected ticks and two pools of ten SGs of *E. canis* infected ticks. While for IDE8 cell line, RNA was obtained from eight uninfected flasks of 25cm^3 and five *E. canis* infected flat-side tubes. Validation of *E. canis* infection was performed by amplification of the 16S rRNA gene for ticks, and *dsb* for tick cells. Interestingly, detection of *E. canis* in *R. sanguineus* ticks was not possible through amplification of *dsb* with qPCR, but only through nested-PCR. On the other hand, amplification of bacteria from IDE8 samples could only be performed with qPCR and not through nested-PCR, suggesting that sample concentration may be the determining factor for technique selection, since parasitemia is significantly higher in the *in vitro* culture than in the tick's SGs. The low quantities of genetic material obtained from SGs of individual ticks difficult the detection of the bacteria, but the preparation of pooled samples also has the disadvantage of not guaranteeing that every tick is indeed infected with *E. canis*, which may dissimulate variations due to impact of infection.

Data normalization was performed by recurring to the reference genes that showed the lowest variation between uninfected and infected tick samples: *16s*, *β-tubulin* and *elf* for *R. bursa*; *β-actin* and *elf* for *R. sanguineus*; *16s, β-tubulin* and *β-actin* for IDE8 cells.

Differential gene expression results between uninfected and infected samples for all four biological systems is represented in Fig. 19, for *gch-I* (Fig. 19, A), *ts* (Fig. 19, B) and *ptps* (Fig. 19, C).

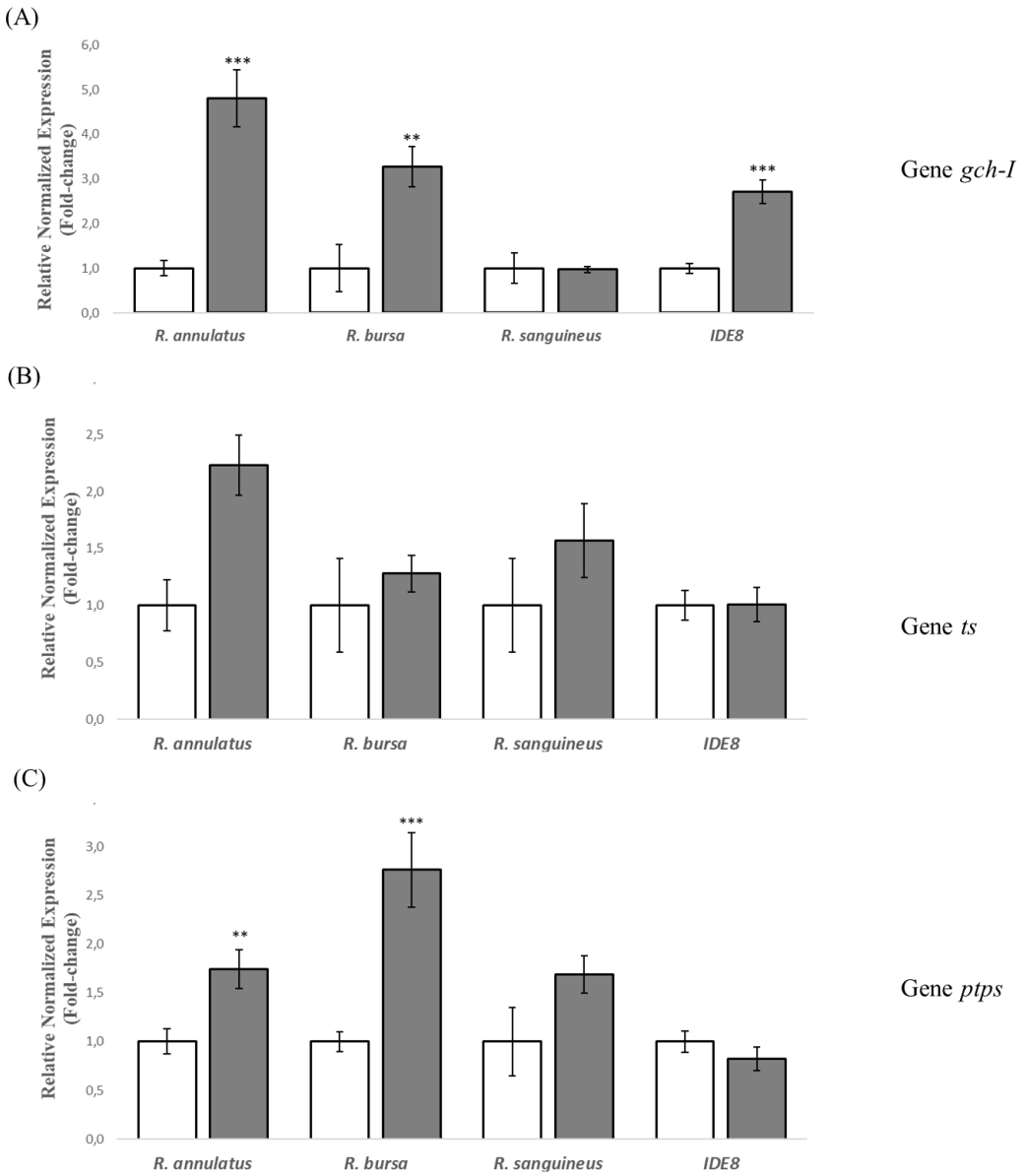


Figure 19 - Differential gene expres NI I ıll biological systems. Relative expression of *gch-I* (A), *ts* (B) and *ptps* (C) genes for the infected (I) samples against the non-infected (NI) controls in the four biological systems: *R. annulatus* - *B. bigemina*, *R. bursa* – *B. ovis*, *R. sanguineus* - *E. canis* and IDE8 – *E. canis*. Significant statistical differences are indicated with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

The *gch-I* gene showed significant upregulation in the *B. ovis*-infected *R. bursa* ticks of about 3.3-fold and *E. canis*-infected IDE8 cells about 2.2-fold in comparison to the corresponding uninfected controls, as seen in Fig. 19, A. The *ts* gene presented no

significant differential expression but showed a tendency for increased expression in the infected samples for the *R. bursa – B. ovis* and *R. sanguineus – E. canis* (Fig. 19, B). The *ptps* gene showed statistically significative upregulation for *B. ovis*-infected *R. bursa* samples, of approximately 2.8-fold (Fig. 19, C). However, for the *E. canis* infected IDE8 cells, expression levels of *ptps* where slightly reduced, contradicting the result obtained for *E. canis* infected *R. sanguineus*, even though neither of these results is statistically significative.

The *in vitro* system seems to replicate the tendencies observed for *gch-I* expression in the *Rhipicephalus* - *Babesia* systems, even though they may differ from the results obtained for *Rhipicephalus* - *E. canis.* These observations bring forward the problems associated with the use of the IDE8 tick cell line for comparison studies with *Rhipicephalus* ticks.

Taking into account these results, the *gch-I* gene was selected for functional analysis since it was the gene with the highest fold-change and the one affected in the majority of the biological systems.

3.6. *In vitro* gene knockdown assay

To better understand the role of *gch-I* in the vector-parasite interface an *in vitro* silencing assay by RNA interference was performed. For RNAi, dsRNA was produced since it was previously suggested to be more efficient than siRNA in tick cells lines (Barry et al., 2013).

Amplification of the *gch-I* gene was performed with primers containing the T7 sequence and a single product was obtained with the approximately expected size of 458 bps (Fig. 20, A).

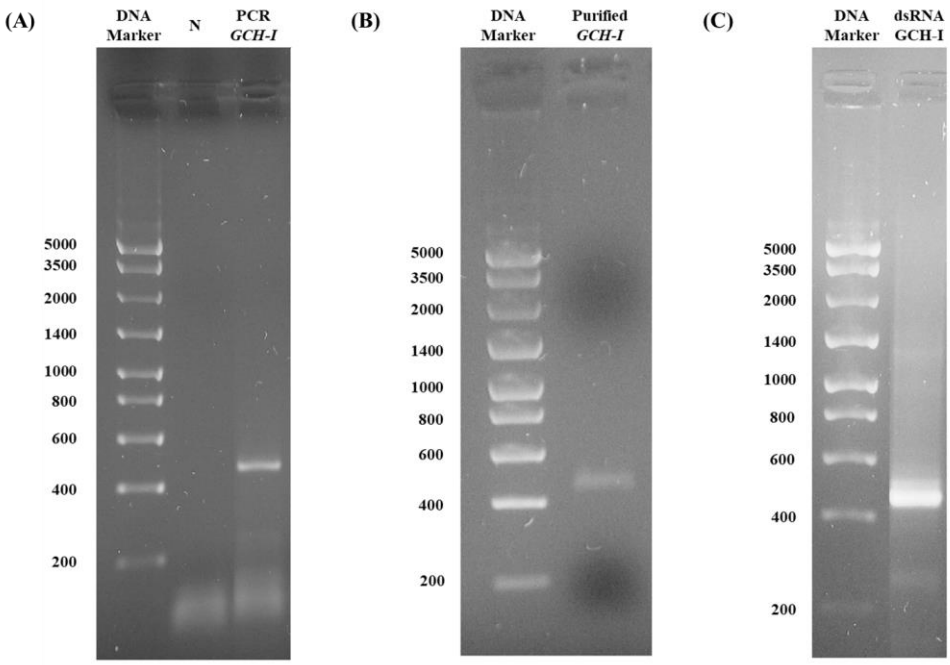


Figure 20 – Products of dsRNA synthesis in electrophoresis gel. (A) PCR product of T7 containing *gch-I* and negative control (N); (B) Purified T7 *gch-I*; (C) dsRNA *gch-I*, band with approximately 458bp. Electrophoresis gels with 1.2% (w/v) agarose and 0.5X TBE. cDNA Marker: NZYDNA Ladder VI.

The purified product presented a single fragment (Fig. 20, B) and the obtained sequence showed 97.4% of identity with *gch-I* (B7PWM4). This fragment served as template for the dsRNA production which, after purification, was analysed on agarose gel (Fig. 20, C) and showed, besides the expected band at 458 bps, several weak bands of small molecular size. Since the template was a single product it was assumed the small bands obtained were degradation fragments of the dsRNA sequence. This protocol allowed the production of nearly 500 ng/µL of dsRNA.

3.6.1. Gene knockdown assessment

For the *in vitro* knockdown assay three experimental groups were defined: uninfected IDE8 cells (Group A); uninfected cells inoculated with *E. canis* at the second time point (Group B); and pre-established *E. canis* infected IDE8 cells (Group C). For each of three groups, cells were inoculated with $5x10^{10}$ molecules/µL of *gch-I* dsRNA. Additionally, two control groups were performed: inoculated with *β2m* dsRNA, which is originated from mouse RNA samples and functioned as an unrelated gene; and non-inoculated where no dsRNA was inoculated, being a "vehicle" control. After distribution

of the IDE8 cells on the 24 well plates, the dsRNAs were inoculated and samples were collected from the three time points: 48 hours (T1), 120 hours (T2) and 160 hours (T3). For each condition, five pseudo-replicates were performed.

Samples were processed as described previously and the expression of *gch-I* gene was measured through qPCR. Data normalization was achieved with *β-actin*, *16s* and *R13A* gene for a M-value of <1, since <0.5 was not obtained. Previously, (see section 3.3) normalization for the *E. canis* - IDE8 system, was performed with *16s*, *β-tubulin* and *β-actin* which returned a M-value < 0.5; however, in the conditions of this assay *β-tubulin* proved variable and inadequate for the analysis. Moreover, *β-actin* and *R13A* have been described as non-variant housekeeping genes for IDE8 cells (Weisheit et al., 2015). The lack of stability observed revealed a higher biological variability that it would be expected for an established cell line, which could be compensated by the increase of pseudo-replicates for each condition and ideally by the selection of new reference genes, more stable in the assay conditions. On the other hand, the IDE8 cell line proved to be sensitive to manipulation and to easily resuspend and die which may also diminish the consistency of the pseudo-replicates.

Expression values were represented in relation with the unrelated control *β2m* and after outlier elimination, silencing efficiency was calculated for all conditions (Table 4 and Fig. 21).

Table 4 - Silencing efficiency of *gch-I* gene, calculated from relative normalized values from samples inoculates with *gch-I* and *β2m* dsRNA, obtained by qPCR analysis**.** (Group A) – Non-infected IDE8 cells; (Group B) – Non-infected IDE8 cells inoculated with *E. canis* at 48 hours; (Group C) – Established *E. canis* infected IDE8 cells; (T1) – 48 hours; (T2) – 120 hours; (T3) – 160 hours.

Silencing Efficiency (%)	**T1**	**T2**	**T3**
Group A	83.2	96.8	97.9
Group B	92.6	86.6	94.5
Group C	90.5	100	100

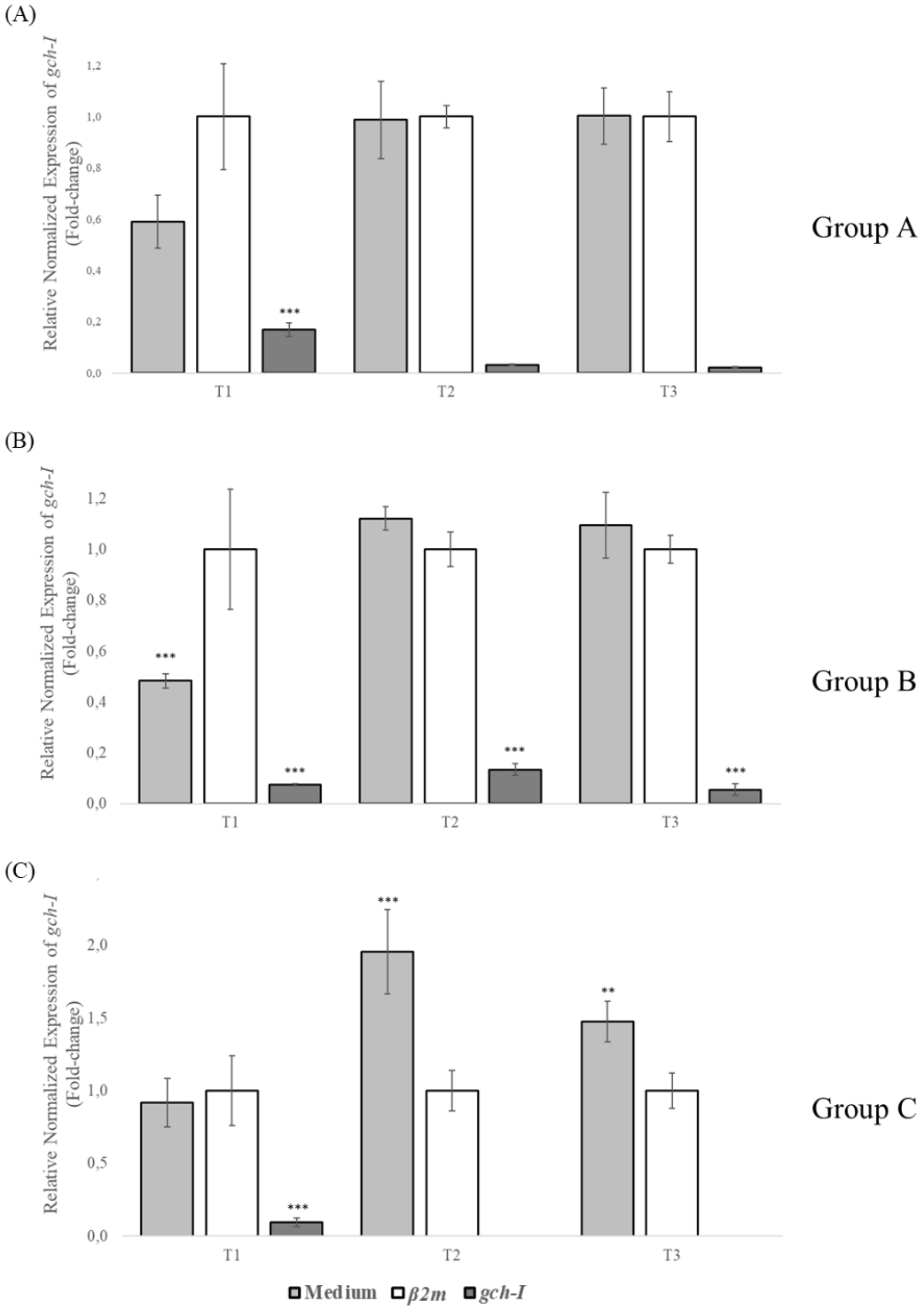


Figure 21 - Gene knockdown assessment. Relative expression of the *gch-I* gene in uninfected IDE8 cells (A), uninfected cells inoculated with *E. canis* at the second time point (B) and pre-established *E. canis* infected IDE8 cells (C). A control with only the addition of medium (light grey bars) was performed. Cells from all three groups were inoculated with *β2m* control gene (white bars) or *gch-I* dsRNA (dark grey bars). Samples were inoculated at 24 hours (T0) and collected at three time points: 48 hours (T1), 120 hours (T2) and 160 hours (T3). All data is relative to the corresponding *β2m* gene control. Significant statistical differences are indicated with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

When amplification of *gch-I* did not occur a 100% silencing efficiency was assumed. Considering these silencing efficiencies, we proceed with the analysis of relative expression of *gch-I* in all conditions (Fig. 21).

First, it would be expected that the *gch-I* relative expression values would be similar between the *β2m* and medium control groups and while this may be true for some samples, the same cannot the observed in other conditions. Like all arthropods, ticks immune systems is only constituted by an innate response (Baxter, Contet & Krueger, 2017), lacking all the mechanisms for an adaptative response and structures like the major histocompatibility complex and β2-microglobulin (coded by *β2m*). As such, no impact would be expected by the addition of dsRNA against this sequence in tick cells. However, statistically significant differences were obtained in Group A in T1, and Group C for T2 and T3. Besides, while in the first condition the expression levels of *gch-I* for *β2m* dsRNA group are higher than in the medium control, the opposite is shown for T2 and T3 from group C. This result suggests an unspecific effect caused by the introduction of dsRNA in the cell (Wang & Carmichael, 2004), that affects the expression levels of *gch-I* leading to: an increase in the first time point for uninfected cells, or an increase for the last two time points for *E. canis* infected cells. For Group C, the fold-change is also higher for T2 than for T3.

3.6.2. *E. canis* quantification

Quantification of *E. canis* was performed to evaluate the role of the *gch-I* gene in the invasion and multiplication of the tick cells.

Absolute quantification of *E. canis* was not possible due to lack of integrity of the DNA samples, which was firstly detected by the quantities and ratios obtained by the spectrophotometric analyse (Nanodrop) and was further confirmed by lack of amplification of the *dsb* and 16S gene, in four random samples from group C (pre-established *E. canis* infected IDE8 cells). No amplification was obtained for any of the four DNA samples, while a fragment of the expected size (near 200 bps) was observed for the positive control - *E. canis* sample from an independent IDE8 cell extraction (Fig. 22).

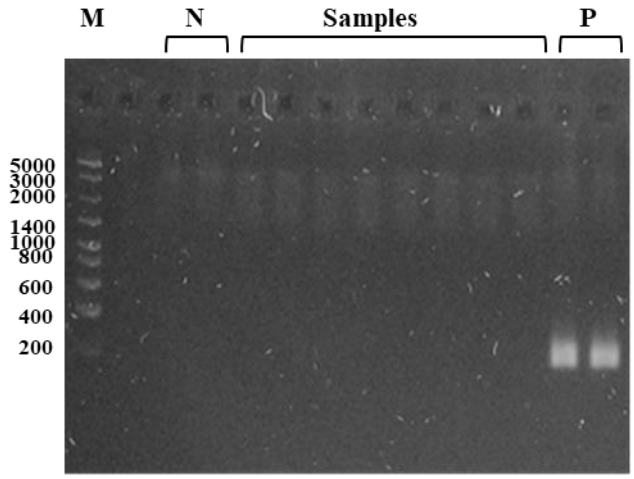


Figure 22 – Amplification of 16S gene. Electrophoresis gel with 1.2% (w/v) agarose and 0.5X TBE of PCR products from the amplification of *16s* gene in four random DNA samples from the *E. canis* infected IDE8 cells. From left to right: marker, NZYDNA Ladder VIII (M); negative controls (N); PCR product for the amplification of 16S gene in DNA samples (Samples); positive control DNA samples from *E. canis* infected IDE8 cells (P).

Since the 16S gene is a tick housekeeping gene this result seems to suggest that a problem occurred during DNA extraction. Therefore, relative quantification was performed resorting to cDNA amplification of the *dsb* gene (Doyle et al., 2005), assuming that differences in this gene expression are proportional to the quantity of *E. canis* alive in the sample. In the *E. canis* genome the *dsb* gene has been described to present one complete and a second incomplete open reading frame (Vidotto, McGuire, McElwain, Palmer & Knowles, 1994). Samples were normalized not only for the same concentration of RNA (250 ng/µL) for cDNA synthesis, but also with two endogenous reference genes from the IDE8 cells, *β-actin* and *r13a* to account for variations in the genetic material, like has been described in other works (Fouzi, Shariff, Omar, Yusoff & Tan, 2010; Santolamazza et al., 2017). The *E. canis* inoculum was set as the calibrator sample and its expression level defined as 1, as such, quantities from other samples are represented relatively to this value (Fig. 23).

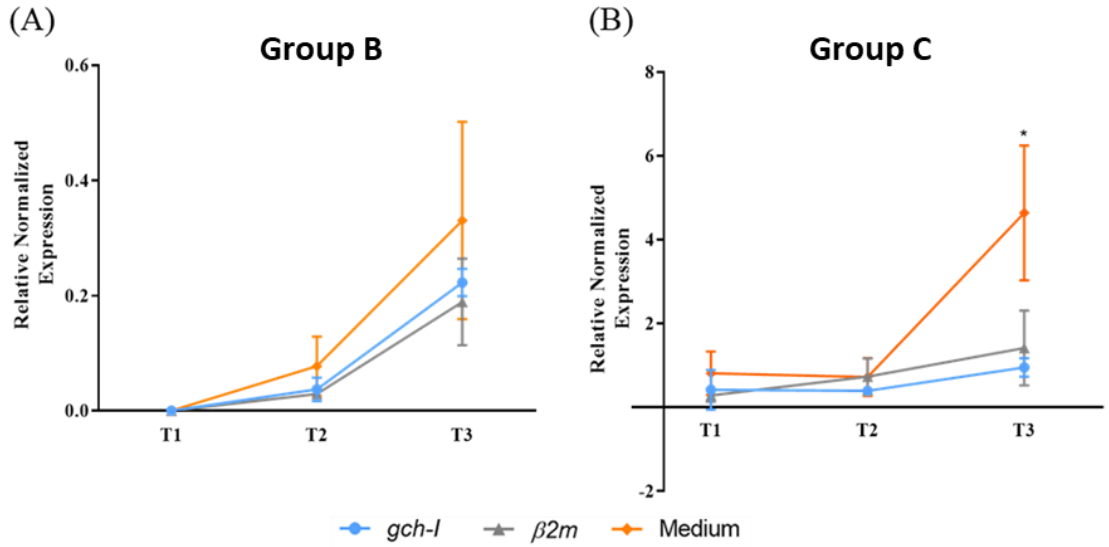


Figure 23 - Relative normalized expression of *dsb* through time. Relative expression of the *dsb* gene for samples of group B - uninfected cells inoculated with *E. canis* at the second time point - and group C - pre-established *E. canis* infected IDE8 cells. Samples were inoculated with: medium (orange, diamond); dsRNA for *β2m* (grey, triangle) or dsRNA for *gch-I* (blue, spheres). Analysis was realized in three time points: 48 hours (T1), 120 hours (T2) and 160 hours (T3). Points in the graph represent the mean and vertical lines the corresponding standard deviation. Significant statistical differences were calculated with the Mann-Whitney test, within the same time point against the *β2m* control samples and are indicated with $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

All samples from group A (uninfected IDE8 cells) showed no amplification of the *dsb* gene confirming the absence of infection. For group B, all samples in T1, also showed no amplification since inoculation was only performed after sample collection in that time point, to understand if the *gch-I* gene had a role in *E. canis* invasion (Fig. 23, A). Amplification was obtained for group B at T2 and T3 and for Group C at all time points (Fig. 21).

Relative expression levels of *dsb* were not significantly different between the groups inoculated with *gch-I* and *β2m* dsRNA for any condition, indicating that silencing this gene had no significant impact on either *E. canis* invasion (Fig. 23, A) or replication (Fig. 23, B), since *dsb* values were similar at the second time point and increased equally over time (Fig.17, B). There was also no statistically significant difference between the medium and the *β2m* control groups, except for one condition (Group C, T3), however the *dsb* relative expression was higher for the medium control, in T2 and T3 in Group B and in T3 for Group C, suggesting that the presence of the foreign genetic material alone may have a unspecific effect which interferes with *E. canis* invasion and replication (Wang & Carmichael, 2004). It was also possible to observe that for Group C, there was no statistically significant increase in the relative *dsb* expression values in samples inoculated

with dsRNA for any time point, even though for Group B, this increase can go from 4 to 6-fold, between T2 and T3. Samples from the medium control from Group C, also showed no increase from T1 to T2 but there was a significant increase from T2 to T3, which was not observed for the other samples. To better understand the phenomena at place, further studies are needed with a higher number of replicates and time points, increasing the concentration of dsRNA inoculated and including a different unrelated gene.

Moreover, cell morphology was also assessed to understand the impact of silencing the *gch-I* gene on cell shape, size and internal structure which may be indicative of other subjacent phenomena (Kiger et al., 2003). In this assay, the observation of Giemsa stained cytocentrifuge smears showed no striking changes in morphologic characteristics in both tick cells and in the bacteria between groups and time-points (Fig. 24).

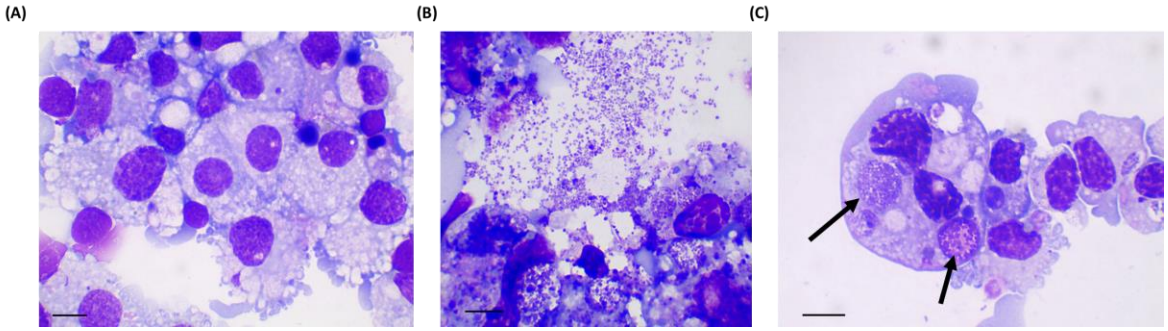


Figure 24 - Light microscopy photographs of IDE8 cells. (A) Uninfected IDE8 cells; (B) *E. canis* infected IDE8 cells and (C) *E. canis* inoculum before purification. Giemsa-stained cytocentrifuge smears were observed with light microscope under x 1000 magnification with immersion oil. Arrows indicate *E. canis* morulae. Scale bars = 20 µm.

This analyse would benefit from a cellular count and viability assay for a quantification of the effect of silencing of *gch-I* gene in the tick cells, however the sensitivity of this cells to manipulation highly alters these parameters.

Even though silencing efficiencies for *gch-I* were high and in some cases of nearly 100%, there is no guarantee this low expression levels correspond to gene knockdown. Protein detection and quantification would be the next step for evaluation of the silencing assay. The use of *gch-I* specific inhibitors would also help to clarify this protein role in the vector-pathogen interface. On the other hand, underlying regulation or compensation mechanism could also diminish the impact of the gene knockdown.

Results suggest that the *gch-I* gene has no impact in the *E. canis* invasion and replication *in vitro*, and therefore more studies are needed to understand if the results obtained *in vitro* are a good indication of what is expected to observe *in vivo* for a better

understand of the potential of this target for the development of transmission blocking or anti-tick vaccines.

4. Conclusions

The increased incidence of TBDs in the last years coupled with the emergence of new tick zoonoses, stresses the need of more efficient strategies of control and eradication. In this context, host vaccination appears the most promising approach to offer protection against ticks and the pathogens they transmit. However, selection of adequate antigens with potential for multi-tick and pathogen protection has proven to be an arduous task, more so for tick species with no sequenced genome available, such as the *Rhipicephalus* spp..

This work was centred in the folate related pathways due to the broad and crucial role these enzymes have on the organisms, extensively studied in mammals but with scarce information available for arthropods or even insects. However, antifolate compounds have already been established has chemotherapeutic agents against *Plasmodium* spp. having been used for treatment and control of malaria (Müller & Hyde, 2013).

Here, was possible to identify five folate related genes in *R. annulatus*, coding for TS, SHMT, AICARFT, GCH-I and PTPS proteins, which showed a general upregulation in the presence of the parasite, which may indicate some molecular modulation. Of these genes, only three were possible to identify in other *Rhipicephalus* species (*R. bursa* and *R. sanguineus*) and in the IDE8 tick cell line – *gch-I*, *ts* and *ptps*. The significant upregulation obtained for the gene coding for GCH-I in the majority of the biological systems tested was the decisive factor for selection of this target for functional analysis. *In vitro* silencing of this gene thought RNAi showed no significant effect on tick cell morphology and on *E. canis* invasion or multiplication. However, further studies should be conducted for validation of gene knockdown. This work increased the available knowledge on folate related pathways in ticks, setting a precedent for the screening of antigens in these pathways.

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Appendix

Supplementary Table 1 - Putative genes and corresponding UniProt ID for the design of primers sequences. All concentration and annealing temperature tested are discriminated by species.

Putative Genes	Protein Encoded (UniProt ID)	Forward primer (5'-3')	Reverse primer (5'-3')	Species	Primer Concentration (µM)	Annealing Temperature (C°)	Length (bp)
GTP cyclohydrolase (gch-I)	B7PWM4 †	GTCAACGATGCYGTGTTC	GCTTGKATSACMACTCCGAC	*R. annulatus*	0,5	55 - 60	275
				R. bursa			244
				R. sanguineus			232
				I. scapularis			225
Alkaline Phosphatase (ap)	B7QGN3	TGACTACCTTCTCGGGCTGT	GACCGTGATCTATGCGACCT	*R. annulatus*	0,5 - 1	48,5 - 62	176
		CATGGCCTACGAACTTCACC	CTGCGTTGCTCAGTTCTACG	*R. annulatus*	0,3 - 1,5	54,8 - 60	200
	B7PF40	CATTCGGGTCACCGTTACTG	TCGCCCGTCCACTCTATATC	*R. annulatus*	0,5 - 1	48,5 - 60	183
	B7P0L3	GTTACAGCCACTCGGGACAT	CGTCCATCATGTTCTTGTCG	*R. annulatus*	0,5 - 1	48,5 - 60	162
		CAAGAACGTGGTGCTCTTCC	TCTCGTAGTTGGCCTTGACC	*R. annulatus*	0,3 - 1	55 - 60,8	230
Fofylpolyglutamate synthase (fpgs)	B7PVJ7	TGAACAACCTGCAGAGCAAC	CAGGATGCTCTCGGTAAAGG	*R. annulatus*	0,5 - 1	48,5 - 60	210
Gamma-glutamyl-hydrolase (gh)	B7PQJ1	ATCAGAACATTCCCCACAGC	TGGCTGTAGCTCACGTCGTA	*R. annulatus*	0,5 - 1	48,5 - 60	150
		ACGGCAGCTATCTCGTATCC	GTTGTAGATCAGGGCTTTGG	*R. annulatus*	0,5 - 1	48,5 - 60	282

6-pyruvoyltetrahydropterin synthase (ptps)	G3MHQ8 †	ACGCGGATTGAATCMTTCAG	TTCTTGTGATCAAGRGCATCC	*R. annulatus*	0,5	55 - 60	198
				R. bursa			195
				R. sanguineus			186
	V5H6X5	AGCAGCATACAGCGTCAGG	TCTCGTGAAGCCGAACTTGG	*I. scapularis*	0,5	56 - 59	113
Sepiapterin reductase (srp)	B7QMY1	CGGTCCTCAGCTACAATCCT	CCCTTAGAATGAACCCGATG	*R. annulatus*	0,5 - 1	48,5 - 60	242
	B7QGH5	GTCGAGACCTGCGATCATTC	TCAGTGTCATCACGGACGTT	*R. annulatus*	0,5 - 1	48,5 - 60	211
	B7PCLO	CCAACACTACGGTTCCGACT	GGATACAGACGGGTTTTCCA	*R. annulatus*	0,5 - 1	48,5 - 60	160
Dihydropteridine reductase (dhrp)	B7QAP3	GCTGTGTACTCCTGGCAACA	GGTCCACTTGAACAGCGTCT	*R. annulatus*	0,5 - 1,5	48,5 - 60	198
Molybdenum cofator biosynthesis protein 1 (mcbpp1)	B7PFI1	CCCTGACTTTGGTGGAAGAA	GTCGGTTATGCTGATCTCG	*R. annulatus*	0,5 - 1,5	48,5 - 60	164
		CTGATTCCGCTGTGTCATCC	TGTCGGTTATGCTGATCTCG	*R. annulatus*	0,3 - 1	55 - 58	211
	B7Q4E2	TGACGCACCTCAACATTAGC	TGCTGCGTAAGTTCCACAAG	*R. annulatus*	0,5	48,5 - 60	173
		CCTTCGGATTTCACTTACGG	GAGGCGTACTTTGTTCACACC	*R. annulatus*	0,5 - 1	48,8 - 59,3	160
Molybdopterin synthase (mpts)	B7Q3T4	ACACGGAGAAGCTGATTTCG	TCTTGACGTTCCACTTGGTG	*R. annulatus*	0,5 - 1,5	48,5 - 60	189
		AAGTGGAACGTCAAGAACG	CAGAAACACTCCTTGTTCTCC	*R. annulatus*	0,5 - 1	48 - 58	218
Molybdenum cofator sulfurase (mcs)	Q9VRAZ X2JFY7	GGAATTCTCTAGATTGGCC	TTCGCCGTGAAKATCACATG	*R. annulatus*	0,5 - 1,5	48 - 58	228

Molybdenum cofactor biosynthesis pathway protein 2 mcbpp2)	B7QGD6	GGACGTTACCCTGACTTTGG	TATTCTGATCTCGCGGCTGA	*R. annulatus*	0,5	48,5 - 60	166
	B7PDW8	ATCTGATCCCGCTCTGTCAT	CCGGACCAATCACCATATCT	*R. annulatus*	0,5	48,5 - 60	213
Molybdopterin synthase large subunit (msls)	B7PDW7	CCGTTCAGCGTAGGAGAAGA	GGTTACGCGCTTCATCAAC	*R. annulatus*	0,5	48,5 - 60	184
Thymidylate synthase (ts)	B7P7E2† L7M0X3	TATGGATTYCAGTGGAGGC	ACRTAGAACTGYGCYARG	*R. annulatus* *I. scapularis*	0,5 - 1	48,5 - 72	206 163
	L7M0X3	ACAGACCCACGATGAATACC	CCGTAGACCCTCTTAGAAACC	*R. annulatus* *R. bursa* *R. sanguineus*	0,3 - 0,7	48 - 58	218 176 172
Methenyltetrahydrofolate cyclohydrolase (mthfc)	B7QFP4 GCJO01004656.1*	TCAGAAAGGACAGGGATAACG	GAGAGTCCATGTTGAGGCAGAG	*R. annulatus*	0,3 - 1,5	55 - 60,8	240
Glutamate formiminotransferase (ftcd)	B7Q6E5	GAGTGGAAGCCAGACTACGG	CCAGGACGTTCACAGAAACC	*R. annulatus*	0,3 - 1	55 - 59,6	250
Methionyl-trna formyltransferase (mft)	B7QKW8	AGAACATCCCATCCTCAAGTTG	GGGTGGACGTTGATCATCCC	*R. annulatus*	0,5	55 - 58,8	220
Aldehyde dehydrogenase (aldh)	B7Q8K1	ATTGTGATAGGCGATCCTCTCG	AGACGAGCATGATTGGAC	*R. annulatus*	0,3 - 1	54,3 - 60,5	236
Aminomethyltransferase (amt)	B7P6X5	CTGACATTCATGCGAAGTGC	CCTGCCCGTAGAGACAGAGC	*R. annulatus*	0,3 - 1,5	48 - 61	223

5-formyltetrahydrofolate cyclo-ligase (mthfs)	B7Q8Q2	CACAGACGCAGAGGTTTCC	ACCACAAGCAAGTCCAAACC	*R. annulatus*	0,3 - 1	54,8 - 61	231
Phosphoribosyl glycinamide formyltransferase (garft)	B7P986	GACTGGTGCATCAAGAACGGC	TTGGTGATGTACGTCTTGGC	*R. annulatus*	0,5	56 - 60,7	218
Phosphoribosyl Aminoimidazole carboxamide formyltransferase (aicarft)	B7PV68	TGTGGAGGTCATCTCAAAGC	CAAGTCGCTTCCTTGTCTCC	*R. annulatus*	0,5 - 1	55 - 58,3	206
Serine hydroxy methyltransferase (shmt)	B7PG87 GANP01012563.1 *	CGAGGCACTGGGATCATGTC	AGTCCCATAAGCCTGTCGTGGG	*R. annulatus*	0,5	57,4 - 61	236
elongation factor (elf)	‡	CGTCTACAAGATTGGTGGCATT	CTCAGTGGTCAGGTTGGCAG	*R. annulatus* *R. bursa* *R. sanguineus*	0,5	62,5	109
β-tubulin	‡	AACATGGTGCCCTTCCCACG	GCAGCCATCATGTTCTTTGC	*I. scapularis* *R. annulatus* *R. bursa*	0,5	58 - 60,5	140
β-actin	‡	GACATCAAGGAGAAGCTTCTGC	CGTTGCCGATGGTGATGC	*I. scapularis* *R. annulatus* *R. sanguineus*	0,3 - 0,8	58	127
16S rRNA	MF946466 *	TTAACTGGGGCGGTTAAAAA	AACATCGAGGTCGCAAACTT	*R. annulatus*	0,5	55 - 60	147
	§	GACAAGAAGACCCTA	ATCCAACATCGAGGT	*I. scapularis* *R. bursa* *R. sanguineus*	0,5	48,2 - 57,5	212

Ribosomal protein L13A (r13a)	¶	GTGGGCTGGAAGTACCAGAA	CTAGCTGAACCTTGGCTTCG	*I. scapularis*	0,5	55 - 63	124
Ribosomal protein L4 (rpl4)	‡	AGGTTCCCCTGGTGGTGAG	GTTCCTCATCTTTCCCTTGCC	*I. scapularis*	0,2 - 0,5	55 - 60,5	152

† Primer designed by alignment of present sequence and transcriptomic sequences
‡ Nijhof et al, 2009
§ Ferrolho et al, 2016
¶ Weisheit et al., 2015
*NCBI entry codes

Supplementary Table 2 - Putative genes with corresponding UniProt ID entries and optimized conditions.

Putative Genes	Protein Encoded (UniProt ID)	Forward primer (5'-3')	Reverse primer (5'-3')	Species	Primer Concentration (µM)	Annealing Temperature (C°)
GTP cyclohydrolase (gch-I)	B7PWM4 †	GTCAACGATGCYGTGTTC	GCTTGKATSACMACTCCGAC	*R. annulatus*	0,5	60
				R. bursa		
				R. sanguineus		
				I. scapularis		
6-pyruvoyltetrahydropterin synthase (ptps)	G3MHQ8 †	ACGCGGATTGAATCMTTCAG	TTCTTGTGATCAAGRGCATCC	*R. annulatus*	0,5	60
				R. bursa		
				R. sanguineus		
	V5H6X5	AGCAGCATACAGCGTCAGG	TCTCGTGAAGCCGAACTTGG	*I. scapularis*	0,5	58
Thymidylate synthase (ts)	B7P7E2 † L7M0X3	TATGGATTYCAGTGGAGGC	ACRTAGAACTGYGCYARG	*I. scapularis*	0,5	59
	L7M0X3	ACAGACCCACGATGAATACC	CCGTAGACCCTCTTAGAAACC	*R. annulatus*	0,5	54
				R. bursa		
				R. sanguineus		
Phosphoribosyl Aminoimidazole carboxamide formyltransferase (aicarft)	B7PV68	TGTGGAGGTCATCTCAAAGC	CAAGTCGCTTCCTTGTCTCC	*R. annulatus*	0,5	57
Serine hydroxy methyltransferase (shmt)	B7PG87 GANP01012563.1 *	CGAGGCACTGGGATCATGTC	AGTCCCATAAGCCTGTCGTGGG	*R. annulatus*	0,5	57,4
elongation factor (elf)	‡	CGTCTACAAGATTGGTGGCATT	CTCAGTGGTCAGGTTGGCAG	*R. annulatus*	0,5	62,5
				R. bursa		
				R. sanguineus	0,4	57,2

β-tubulin	‡	AACATGGTGCCCTTCCCACG	GCAGCCATCATGTTCTTTGC	*I. scapularis*	0,5	60,5
				R. annulatus		
				R. bursa		
				R. sanguineus	0,4	58
β-actin	‡	GACATCAAGGAGAAGCTTCTGC	CGTTGCCGATGGTGATGC	*R. annulatus*	0,5	60,5
				I. scapularis		58
				R. sanguineus	0,8	
16S rRNA	MF946466 *	TTAACTGGGGCGGTTAAAAA	AACATCGAGGTCGCAAACTT	*R. annulatus*	0,5	56
	§	GACAAGAAGACCCTA	ATCCAACATCGAGGT	*I. scapularis*	0,5	57
				R. bursa		56
				R. sanguineus	0,8	57,2
Ribosomal protein L13A (r13a)	¶	GTGGGCTGGAAGTACCAGAA	CTAGCTGAACCTTGGCTTCG	*I. scapularis*	0,5	60

† Primer designed by alignment of present sequence and transcriptomic sequences
‡ Nijhof et al, 2009
§ Ferrolho et al, 2016
¶ Weisheit et al., 2015
*NCBI entry code

Supplementary Table 3 - List of primers used for double-stranded RNA synthesis.

Putative Genes	Protein Encoded (UniProt ID)	Forward primer (5'-3')	Reverse primer (5'-3')	Primer Concentration (µM)	Annealing Temperature (C°)	Length (bp)
GTP cyclohydrolase (gch-I)	B7PWM4 †	ACGACGAGATGGTCATTGTG	AGCGTCGTGTCCCACTCTT	0,5	55-64	461
β-2-microglobulin (β2M)	P01887	CACCCCCACTGAGACTGATACA	AATTAGGCCTCTTTGCTTTACCA	0,5	64	450

† Primer designed by alignment of present sequence and transcriptomic sequences
Note: All primers contained T7 promoter sequences (5´-TAATACGACTCACTATAGGGTACT-3´) at the 5´end